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- (71) Applicant (*for all designated States except US*): **EIRX THERAPEUTICS LIMITED** [IE/IE]; Cork Airport Business Park, Kinsale, Cork (IE).
- (72) Inventors; and
- (75) Inventors/Applicants (*for US only*): **COTTER, Tom** [IE/IE]; Eirx Therapeutics Limited, Cork Airport Business Park, Kinsale Road, Cork (IE). **HAYES, Ian** [GB/GB]; Eirx Therapeutics Ltd, Cork Airport Business Park, Kinsale Road, Cork (IE). **MURPHY, Finbarr** [IE/IE]; Eirx Therapeutics Ltd, Cork Airport Business Park, Kinsale Road, Cork (IE). **SEERY, Liam** [IE/IE]; Eirx Therapeutics Ltd, Cork Airport Business Park, Kinsale Road, Cork (IE).
- (54) Title: **SCREENING METHOD**
- (57) Abstract: The present invention relates to a method for identifying a gene product which modulates the transition of a cell between a non-apoptotic state and an apoptotic state, comprising the steps of: (a) exposing the cell to an inhibitor of GM-CSF mediated inhibition of apoptosis; and (b) exposing the cell to one or more agents which increase tyrosine phosphorylation; and (c) placing the cell in conditions which permit it to undergo spontaneous apoptosis; and (d) monitoring the level(s) of expression of the one or more gene products in the cell; and (e) identifying gene product(s) whose expression has been increased, decreased or modified as a result of performing steps (a) to (d).
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**WO 02/04657 A2**

### Screening Method

The present invention relates to a method for identifying genes, which are involved in  
5 the modulation of apoptosis in cells. In particular, the invention relates to a novel  
method for identifying and characterising those genes, and the molecular mechanisms  
involved in the GM-CSF mediated inhibition of cell death.

Programmed cell death or apoptosis is a genetically programmed process by which  
10 cells die under both physiological and a variety of pathological conditions (Kerr et al,  
Br. J. Cancer, 26, 239-257, 1972). It serves as the counter-balancing force to mitosis  
during adult life and is a major contributor to the sculpting of physiological structures  
during the many processes of development (Wyllie et al, Int. Rev. Cytol, 68, 251-305,  
1980). It is characterised by a number of well-defined biochemical hallmarks. These  
15 include DNA fragmentation, caused by the activation of an endogenous endonuclease  
enzyme (Wyllie, Nature, 284, 555-556, 1980; Enari et al., Nature, 391, 43-50, 1998).  
The result is a DNA ladder pattern that can be readily visualised in agarose cells.  
Coupled with DNA fragmentation is cell shrinkage (Wesselbory et al., Cell Immunol.  
148, 234-41, 1993) where water is actively extruded from the cell. The apoptotic cell  
20 then undergoes fragmentation into apoptotic bodies that are engulfed by neighbouring  
cells or cells of the reticuloendothelial system.

The number of factors which are known to induce survival in particular cell types is  
ever increasing (e.g. IL2, IL3, IL4, IL5, IL8, GM-CSF, insulin like growth factor 1,  
25 NGF, VEGF, PDGF, SCF, LIF, EGF etc.). Many of these survival factors appear to  
share a commonality in the survival pathway (Datta SR et al. *Genes and Development*  
13: 2905-2927, 1999). For an extracellular stimuli, to confer survival on a cell, it must  
inhibit the endogenous apoptotic machinery. The model predicts that there is a series  
of temporal events that occur upon survival factor/ receptor interaction. The first of  
30 these is tyrosine phosphorylation at the plasma membrane due either to intrinsic  
receptor tyrosine kinase activity (e.g. the insulin growth factor 1 receptor), or

indirectly coupled to tyrosine kinases or alternatively directly coupled to several transmembrane G protein-coupled receptors.

Blood neutrophils have relatively short lives with greater than 80% of them apoptosing within the first 24 hours. Apoptotic neutrophils are phagocytosed by macrophages via thrombospondin and macrophage CD36/ vitronectin receptor (Savil 1992, Clinical Science 83, 649-55, Savil et al. 1993, Immunology Today 14,131-136) and thus prevent release of potentially lethal cocktail of enzymes in the host, should the neutrophil undergo necrosis. However, certain inflammatory environments favour the survival of neutrophils. In vitro, several cytokines including GM-CSF, IL-1, IL-2, IL-8 and IFN $\gamma$  can delay neutrophil apoptosis (Brach et al, 1992, Blood 80, 2920 –2924; Calotta et al 1992, Blood 80, 2012-2020, Lee et al 1993, J Leuk Biol 54, 283 – 388, Pericle et al 1994, Eur. J. Immunol24, 440 – 444, Get ref for IL8). Furthermore, inflammatory proteins e.g. C5A and bacterial products e.g. LPS) have also been shown to inhibit apoptosis. These findings together with other results demonstrating that the presence of either actinomycin D or cycloheximide can promote apoptosis in PMN (Whyte et al, 1991, Clin Sci. 80:5p) suggests a role for active gene expression and translation in control of PMN apoptosis. Moreover, other investigators have shown that NF $\kappa$ B regulated genes seem to play a critical role in preventing apoptosis induced by TNF $\alpha$ , since inhibition of this transcription factor using the fungal metabolite Gliotoxin, induces rapid apoptosis (Ward et al. 1999, J. Biol. Chem. 274. 4309-4318). The same investigators also demonstrated that blocking NF $\kappa$ B with Gliotoxin removes the anti-apoptotic effect of LPS. Yoshida et al have identified an alternative mode of action of gliotoxin. These investigators demonstrated that gliotoxin inhibited NADPH oxidase and consequently prevented the onset of superoxide generation by human neutrophils in response to phorbol myristate. (Yoshida et al Biochem Biophys Res Commun 2000, 268(3) 716-23).

Granulocyte macrophage colony-stimulating factor (GM-CSF) is known to inhibit PMN apoptosis both in vitro and in vivo (Cox et al. 1992, Am. J. Respiratory Cell Mol Biol. 7, 507; Chintinis et al 1996, J. Leuk Biol 59:835). One consequence of GM-CSF treatment of PMN is a time and dose dependent tyrosine phosphorylation event within

the cell (McCall et al., 1991, Blood 78(7) 1842-52). That tyrosine phosphorylation is implicated in the regulation of apoptosis has been demonstrated (Simon et al 1995, Int. Arch Allergy Immunol. 107, 338-339). These workers demonstrated that the effect of GM-CSF on granulocyte cell death could be attenuated by the tyrosine kinase inhibitor  
5 genestein, suggesting that increases in tyrosine phosphorylation are essential to inhibit cell death. To further analyse a role for tyrosine phosphorylation, the authors increased levels of tyrosine phosphorylation using the protein- phosphatase inhibitor phenylarsine oxide (PAO). Similar to GM-CSF, treatment of the cells with PAO is followed by a large increase in tyrosine phosphorylation and matched inhibition of  
10 apoptosis. Inhibitors of tyrosine phosphorylation (Genestein and Herbimycin A) reversed the effects of PAO on tyrosine phosphorylation and neutrophil apoptosis.

Furthermore, Wei et al (J. Immunology 1996,157, 5155-5162) suggested specificity in the anti-apoptotic signalling pathway by showing that GM-CSF inhibition of  
15 programmed cell death did not appear to be related to known proteins associated with cell survival i.e. p53, cdc2, Rb, and Bcl-2. However GM-CSF did induce a rapid activation of Lyn, a src family tyrosine kinase, and Lyn antisense treatment of neutrophils reversed the survival promoting effect of GM-CSF. Other investigators have demonstrated that GM-CSF selectively induced tyrosine phosphorylation of  
20 Extracellular Signal-Related kinase (ERK), a member of microtubule associated protein kinase (MAPK) family (Yuo et al. 1997, BBRC 235, 42- 46). Al-Shami et al. (Blood 1997, 89(3) 1035-1044) has shown that GM-CSF induces both a time and concentration- dependent increase in the level of tyrosine phosphorylation of the PI-3-kinase regulatory subunit p85, possibly via lyn kinase. In corroboration of these  
25 results, Klein et al. (J. Immunol. 2000, 164, 4286-4291), using pharmacological inhibitors of signal transduction, further demonstrated a role for PI 3-kinase and ERK. These investigators showed that GM-CSF caused a rapid phosphorylation of the protein Akt, a substrate for PI 3-kinase. Akt phosphorylation is in turn associated with phosphorylation of BAD, a pro-apoptotic member of the Bcl-2 family. The authors  
30 hypothesised that this phosphorylation resulted in disengagement of Bad with anti-apoptotic family members of Bcl-2 family, allowing them to prevent neutrophil apoptosis.

The link between GM-CSF and tyrosine phosphorylation and inhibition of programmed cell death is presently unknown. Previously, prolonged survival of PMN caused by inhibition of apoptosis is observed in bcl-2 transgenic mice (Lagasse and Weissman, 1994, J. Exp. Med. 179 1047). This result is surprising since normal peripheral blood neutrophils are negative for bcl-2 (Wei et al. J. Immunology 1996,157, 5155-5162), however it does show that targets for the Bcl-2 family of apoptosis associated proteins can control PMN apoptosis. Weinman et al. (1999, Blood, 93, 3106-3115) investigated the role of other members of the Bcl-2 family in regulating PMN apoptosis. The authors cultured PMN for 0, 2,6 or 22h in the presence of TNF $\alpha$  (pro-apoptotic) or GM-CSF or are left untreated. Fresh, unstimulated PMN showed a high level of expression of Bcl-XL that gradually decreased as the culture proceeded, suggesting that loss of this protective protein may play a role in spontaneous apoptosis. The reduction of Bcl-XL in the presence of TNF $\alpha$  is much stronger when compared to control cells. GM-CSF did not alter the effect of Bcl-XL. Next the investigators examined expression of Bax- $\alpha$ , a proapoptotic member of the BCL-2 family. Results showed that GM-CSF induced a down regulation of Bax- $\alpha$  when compared to control cells, suggesting that the down-regulation of this death promoting is involved in PMN survival mediated by GM-CSF. The authors concluded that GM-CSF seems to promote survival by modulating the Bax- $\alpha$ / Bcl-XL ratio via down regulation of Bax- $\alpha$ . Furthermore, the authors suggested that inhibition of apoptosis by GM-CSF might be due to a caspase 3 regulation since no further reduction of apoptosis is observed, above that already seen, when PMN are stimulated GM-CSF after inhibition of caspase-3 with its inhibitor Z-DEVD-FMK.

Other members of the BCL-2 family have also been implicated in neutrophil apoptosis. Expression of myeloid cell leukaemia 1 (MCL1), another viability-promoting family member, has been shown to decrease during neutrophil apoptosis but increases in response to GM-CSF and LPS, suggesting a link with PMN survival, (Moulding DA, Quayle JA, Hart CA, Edwards SW, Blood 1998; 92(7): 2495-502). Neutrophils also

express mRNA for A1, another BCL2 homologue with anti-apoptotic properties (Chuang PI, Yee E, Karsan A, Winn RK, Harlan JM, Biochem Biophys Res Commun 1998; 249(2): 361-5). The authors demonstrated that agonists that promote cell survival (e.g. LPS and G-CSF) up-regulated the message for this protein. Moreover, 5 neutrophil apoptosis is enhanced in mice that lack A1-a, a subtype of the A1 gene, and LPS- induced inhibition of apoptosis is abolished. However in these mice TNF $\alpha$  induced apoptosis is unchanged, which suggest that A1 is involved in regulating some but not all neutrophil apoptotic pathways (Hamasaki A, Sendo F, Nakayama K, Ishida N, Negishi I, Nakayama Ki, Hatakeyama S, J Exp Med 1998; 188(11):1985-92).

10

Thus although a few pieces of the apoptotic pathway are in place, key events regulating PMN apoptosis and growth factor induced survival factors are still poorly understood as indeed they are in other cell types.

## 15 **Summary of the Invention**

The present inventors hypothesised that if a model was designed such that the course of apoptosis following induction and its subsequent inhibition by GM-CSF was functionally characterised both in terms of mechanism and time course, then changes, 20 or patterns of changes, in the 'early' regulatory events could be studied. Studies of changes in expression of those genes involved in these early events, would lead to the identification of potential therapeutic targets. Thus, the present inventors have set out to provide a model system for the GM-CSF mediated inhibition of apoptosis such that those genes involved in the early regulatory events of apoptosis, can be identified and 25 characterised.

The control of apoptosis represents a significant therapeutic target, since many diseases are due to defects in this process. Many physiological factors prevent cell apoptosis. For example cytokines or growth factors inhibit death through apoptosis. 30 There is an acute need to identify the genes that regulate this process. In other words, if one identifies a gene that prevents apoptosis, then this gene/gene product or its

function can be blocked by a drug and apoptosis allowed to occur. To-date many of the genes found have certain fundamental flaws e.g. they act late in the process, after the cell has committed to a death programme, or they are ubiquitous, that is they are not restricted to a particular cell type. The ideal targets to control apoptosis act early in the process and are restricted to a particular cell type.

The inventors have discovered that GM-CSF inhibits death through apoptosis by the regulation of 'effector genes' that control the process of apoptosis. A signal acts through a signal transduction cascade and is associated with significant changes, or patterns of changes, in gene expression in the cell. If model discovery assays are configured to target these 'early' regulatory events occurring in the inhibition of apoptosis it is possible to identify the key genes to control apoptosis.

The present inventors have further discovered that if a model is designed such that the inhibition of apoptosis by GM-CSF is itself inhibited by a drug, then changes, or patterns of changes can be targeted by clustering those changes that are common and both increase and/or decrease depending on the treatment. For example, a change that is a 'decrease' following induction of apoptosis is a candidate target gene. Moreover, a change that is additionally an 'increase' following inhibition of apoptosis by GM-CSF has a higher probability of being a target gene because its regulation shows increased correlation with the process. Likewise, a change that is further a 'decrease' following inhibition of GM-CSF inhibitory effect has a yet higher probability of being a target gene because its regulation shows increased correlation with the process. This concept is illustrated by Figure 1, which shows correlation between induction of apoptosis, inhibition of apoptosis by GM-CSF and inhibition of GM-CSF-mediated survival by an inhibitor e.g. gliotoxin. The central shaded region indicates changes associated with all three treatments.

Thus, in a first aspect the present invention provides a method for identifying a gene product which modulates the transition of a cell between a non-apoptotic state and an apoptotic state, comprising the steps of:

(a) exposing the cell to an inhibitor of GM-CSF mediated inhibition of apoptosis; and



- (b) exposing the cell to one or more agents which increase tyrosine phosphorylation;  
and  
(c) placing the cell in conditions which permit it to undergo spontaneous apoptosis;  
and  
5 (d) monitoring the level(s) of expression of the one or more gene products in the cell;  
and  
(e) identifying gene product(s) whose expression has been increased, decreased or  
modified as a result of performing steps (a) to (d).
- 10 In a preferred embodiment of this aspect of the invention, an additional step is  
introduced of; determining the level(s) of expression of one or more gene product(s) in  
a cell to establish a reference expression level;

The present inventors have demonstrated that gliotoxin blocks GM-CSF inhibition of  
15 apoptosis. This effect appears selective to GM-CSF, in that gliotoxin does not itself  
increase the rate of neutrophil apoptosis. The effect of Gliotoxin is mediated, at least in  
part, via NF $\kappa$ B and/or by a mechanism involving NAD(P)H. Accordingly, in a  
preferred embodiment of this aspect of the invention, gliotoxin is used to inhibit GM-  
CSF inhibition of apoptosis.

20 The DNA binding ability of NF $\kappa$ B may be modified by administration of agents  
known to increase levels of NF $\kappa$ B. Examples of suitable reagents includes the addition  
of LPS, TNF $\alpha$ , transfection of p65 or p50 components of NF $\kappa$ B. It should be  
understood that this list is by no means exhaustive. The DNA binding ability of NF $\kappa$ B  
25 may be decreased by agents known to inhibit the migration of NF $\kappa$ B into the nucleus,  
for example, CAPE (Caffeic acid phenethyl ester), lactacystin, and the transfection of  
I $\kappa$ B analogues.

In addition, the inhibitory effect of GM-CSF on neutrophil apoptosis, may be blocked  
30 by agents known to inhibit the function of NADPH oxidase. Suitable agents include,

but are not limited to any one or more of the following: phenyl arsine oxide, diphenylene iodonium. One skilled in the art will be aware of other suitable inhibitors.

The intracellular GM-CSF concentration may be increased by administering  
5 exogenous GM-CSF, or by inducing endogenous GM-CSF production in the cell. The latter may be performed by methods known to those skilled in the art, and will be described infra.

Agents suitable to increase tyrosine phosphorylation include phenylarsine oxide  
10 (PAO). Tyrosine phosphorylation mimics the effect of GM-CSF. Accordingly, in an alternative embodiment of this aspect of the invention, GM-CSF may be used to substitute for agents which increase tyrosine phosphorylation in step (c) of the method of the present invention..

15 The intracellular tyrosine phosphorylation concentration may be altered by administering agents which inhibit tyrosine kinase, for example Genestein, or agents which inhibit tyrosine phosphatases, for example Phenylarsine Oxide. Those skilled in the art will be aware of other suitable agents.

20 It has been found that exposure of the cell to GM-CSF leads to inhibition of apoptosis in the cell at a different rate than occurs under identical conditions but in the absence of GM-CSF. The assay of the invention may be configured to identify gene products that accelerate or retard the induction of apoptosis. In a preferred embodiment of this aspect of the invention, the assay detects gene products that inhibit the induction of  
25 apoptosis.

Likewise, it has been found that exposure of the cell to agents which increase the tyrosine phosphorylation leads to inhibition of apoptosis in the cell at a different rate than occurs under identical conditions but in the absence of increased tyrosine  
30 phosphorylation. The method of this aspect of the invention may be configured to identify gene products that accelerate or retard the induction of apoptosis.

In addition, it has been found that exposure of the cell to agents which increase the NF $\kappa$ B–DNA binding leads to inhibition of apoptosis in the cell at a different rate than occurs under identical conditions but in the absence of increased NF $\kappa$ B–DNA binding. The assay of the invention may be configured to identify gene products that accelerate  
5 or retard the induction of apoptosis. Advantageously, the assay detects gene products that inhibit the induction of apoptosis.

The method of the invention is applicable to the discovery of gene products, and the genes encoding them, which are involved in apoptosis. The gene products may be  
10 polypeptides and/or RNAs. "Polypeptide" herein refers to any peptide comprising two or more amino acids, whether comprising a single domain or multiple domains, and includes multi-subunit proteins, which are cellular gene products. RNA gene products include ribozymes, antisense RNA molecules and/or mRNA molecules. Advantageously, the gene products are natural gene products, that is they are encoded  
15 by naturally-occurring genes in the cell being investigated and are assembled in the cell using natural components such as amino acids or nucleotides. However, the invention also encompasses screening for gene products encoded by genes that are not endogenous to the cell being investigated. Such genes may be, for example, heterologous genes from other cells or organisms, artificial genes encoding  
20 polypeptides comprising domains from different sources or composite RNA molecules, and wholly or partially randomised genes encoding repertoires of polypeptide or nucleic acid gene products.

Levels of gene expression may be determined in any appropriate manner. Preferably,  
25 the invention comprises the measurement of protein production by mRNA translation, and is configured to detect increases or decreases in the rate or amount of mRNA translation. The invention may also be configured to detect changes in post-translational processing of polypeptides or post-transcriptional modification of nucleic acids. For example, the invention may be configured to detect the phosphorylation of  
30 polypeptides, the cleavage of polypeptides or alternative splicing of RNA, and the like. Levels of expression of gene products such as polypeptides, as well as their post-translational modification, may be detected using proprietary protein assays or

techniques such as 2D polyacrylamide gel electrophoresis. Polypeptide or nucleic acid populations may be assessed individually, or together, in order to identify candidate gene products.

- 5 Advantageously, expression levels are assessed by measuring gene transcription; preferably carried out by measuring the rate and/or amount of specific mRNA production in the cell. A preferred embodiment of this aspect of the invention involves the use of arrayed oligonucleotide probes capable of hybridising to mRNA populations. Differences in hybridisation patterns of different mRNA populations may  
10 be used to identify genes that are differentially expressed in the two populations. The arrayed oligonucleotide probes are advantageously derived from cDNA or EST libraries, and represent genes that are expressed by the cells under investigation.

- As used herein, the terms "oligonucleotide" and "polynucleotide" are equivalent, and  
15 imply no limitation as to maximum or minimum length.

A reference expression level, once established for a given cell type, may be used for repeated screens, thus facilitating the performance of repeated rounds of screening.

- 20 Cells useful in the method of the invention may be from any source, for example from primary cultures, from established cell lines, in organ culture or *in vivo*. Cell lines useful in the invention include fibroblast cell lines, carcinoma cell lines such as neuroblastoma cell lines and cell lines of haematopoietic origin. Preferred are primary cultures of neutrophils or cells having neutrophil characteristics, for example HL-60  
25 cells.

Increases in the NF $\kappa$ B DNA binding can be achieved by treatment of the cells with LPS, TNF $\alpha$ , and p65/p50 transfections.

- 30 A number of methods are known in the art for monitoring the onset of apoptosis. These include morphological analysis, MTT assay, Crystal Violet, DNA ladder

formation, externalisation of membrane phospholipid phosphatidylserine and caspase activation analysis.

The ability of GM-CSF to inhibit apoptosis is preferably confirmed by monitoring the onset thereof according to one or more of the above methods. Moreover, GM-CSF inhibitors, such as anti-GM-CSF antibodies may be used to further substantiate the role of GM-CSF. Techniques for monitoring the onset of apoptosis and antibody technology will be familiar to those skilled in the art.

The ability of increased tyrosine phosphorylation to inhibit apoptosis is preferably confirmed by monitoring the onset thereof according to one or more of the above methods. Moreover, agents that inhibit tyrosine phosphorylation, such as Genestein may be used to further substantiate the role of increased intracellular tyrosine phosphorylation levels.

Genes regulated in these models following modulation of apoptosis include genes that 1) are 'effector' genes involved in the cells defence mechanisms aimed at preventing apoptosis (anti-apoptotic genes) and thus represent therapeutic targets, 2) make up aspects of the apoptosis and/or GM-CSF signal cascade and thus represent therapeutic targets, 3) initiate the process of apoptosis (pro-apoptotic genes) and thus represent therapeutic targets, and 4) are associated with the processes of apoptosis and defence that will aid in the understanding of key pathways, processes and mechanisms that may subsequently lead to the identification of therapeutic targets.

In a further aspect, the present invention provides the use of gliotoxin to inhibit the GM-CSF mediated inhibition of apoptosis.

In a final aspect, the present invention provides a system for modelling GM-CSF mediated inhibition of apoptosis in a cell comprising the steps of:

- (a) the provision of a population of cells;
- (b) exposing the cell to an inhibitor of GM-CSF mediated inhibition of apoptosis; and

- (c) exposing the cell to an agent which increases tyrosine phosphorylation and/or; exposing the cell to GM-CSF; and
- (d) placing the cell in conditions which allow it to undergo spontaneous apoptosis; and
- (e) analysing the gene expression of the cell population; and
- 5 (f) assessing the onset of apoptosis in said cell population.

#### **Brief description of the figures**

**Figure 1** shows the correlation between the induction of apoptosis, inhibition of apoptosis by GM-CSF and the inhibition of GM-CSF-mediated survival by an  
10 inhibitor, for example gliotoxin. The central shaded region indicates changes associated with all three treatments.

**Figure 2** shows the caspase activity present in the supernatant fraction of human neutrophils which have undergone spontaneous apoptosis. The activity is measured  
15 using absorbance at 405nm and normalised per mg of protein. Figure 2 shows the caspase activity during the incubation period. There is a dramatic increase between 8 and 20h of culture.

**Figure 3** shows a morphological determination of apoptosis. Cells are removed from  
20 culture and centrifuged at 300g for 10 minutes at 4<sup>0</sup>C. The pellet is kept on ice, washed in ice cold hanks buffer and then re-suspended in cell lysis buffer. Details are given in example 1. Apoptosis as measured by morphology, increased from approximately 6 hours following isolation of the cells. There is a large increase in the number of cells undergoing spontaneous apoptosis between 6-8 hours resulting close  
25 to 50% of the cells showing apoptotic morphology at 8 hours.

**Figure 4** shows that neutrophil spontaneous apoptosis is accompanied by the endogenous of ROS, in the form of O<sub>2</sub><sup>-</sup> and H<sub>2</sub>O<sub>2</sub>. Experimental details are given in  
Example 1.

30

**Figure 5** shows the dose responsiveness of the anti-apoptotic effect of GM-CSF. Optical densities are read at 570 using a plate reader. The results indicate a direct

correlation between survival and concentrations of GM-CSF added to the culture medium.

**Figure 6** shows a determination of the temporal relationship between GM-CSF addition and survival. A time course of GM-CSF additions to neutrophils is carried out in order to determine up to what stage GM-CSF can induce survival in neutrophils. The method is described in detail in example 1. Optical densities are read at 570 using a plate reader. The results demonstrate that GM-CSF can be added to neutrophils as late as 5 hours post isolation and not result in any discernible loss in the protective effect of this cytokine. The data also indicates that the commitment to die for the vast majority of neutrophils is later than 5 hours post isolation.

**Figure 7** shows timed additions of anti GM-CSF antibody to neutrophils in order to determine how immediately the anti-apoptotic effects induced by GM-CSF occur. The method is described in detail in example 1. Optical densities are read at 570nm using a plate reader. Comparable survival is obtained when addition of anti GM-CSF is delayed for 3 hours following isolation as to when no neutralizing antibody is added. In contrast, when neutralizing antibody is added before the 3 hours, survival is directly linked to the addition time. This indicates that the survival mediated by GM-CSF is not immediate and that cells must be exposed to the cytokine for a defined period of time before survival is conferred.

**Figure 8** shows the role of intracellular phosphorylation in delaying the apoptotic process in neutrophils. The addition of low doses of the phosphorylation inhibitor Phenylarsine Oxide (PAO) to neutrophils increases the survival of its cells after 24 hours in culture. The method is described in example 2. Optical densities are read at 570 nm using a plate reader. The results show that intracellular tyrosine phosphorylation plays a pivotal role in the protective effects on anti-apoptotic agents described in the study.

30

**Figure 9** shows that the fungal metabolite gliotoxin blocks the GM-CSF in the inhibition of neutrophil apoptosis. The method is as described in example 3. Optical

densities are read at 570nm using a plate reader. Gliotoxin effectively blocks the GM-CSF mediated inhibition of neutrophil apoptosis. The blocking effect is not seen when the inactive analogue of gliotoxin, methogliotoxin is added with GM-CSF. No increased neutrophil apoptosis is seen with the addition of gliotoxin alone to isolated  
5 neutrophils, demonstrating that the effect is specific to, and limited to, a reversal of the protective effects of GM-CSF.

**Figure 10** shows the use of microarray to confirm the differential expression of SSH clones. A detailed method is described in example 4. Approximately 100 colonies  
10 are isolated from each reciprocal SSH cDNA library. cDNA insert sequences are amplified by PCR, and spotted onto microarray filters as described above. Duplicate filters are hybridised radio-labeled cDNA probes generated from the reciprocal RNA material used to generate each SSH library. An analysis of the filters identifies which cDNA clones are differentially expressed.

15

**Figure 11.** Positive clones from the first round of screening described in example 4 and fig 10 were subsequently submitted to GeneScreen for preparation of a micro array. This array is then hybridised against radiolabelled probes of choice to further analyse the expression profiles of the mRNAs corresponding to the isolated DNA  
20 sequences.

**Figure 12** shows the use of a STORM phosphoimager to quantitatively image positive signals across the filter arrays. Hybridised filters are wrapped in plastic wrap and exposed to a low energy phosphoimaging screen. The screen is then placed on a  
25 phospho imager and the gel image captured by scanning at a resolution of 50 microns. Full experimental details are given in Example 4.

**Figure 13.** The captured image file is then analyzed using software such as ArrayVision, the program contains facilities as spot detection and quantification, and  
30 background detection and quantification. The data is then exported to a text file for further analysis.



**Figure 14.** Following cluster analysis fold-change data can be difficult to interpret owing to either a very large data set and/or a wide range in fold change values. The interpretation of this data may be simplified using codes or combined codes. The use of combined codes can greatly simplify the Cluster analysis and subsequent visualization.

**Figure 15** shows that GM-CSF inhibition of neutrophil apoptosis is associated with significant changes in global mRNA expression. Microarray analysis of gene expression changes associates with GM-CSF inhibition of neutrophil apoptosis was carried out using LifeGrid microarray filters. The details method is described in example 5. A significant number of genes are down regulated following addition of GM-CSF and these also represent candidate target genes.

**Figure 16.** A comparison of co-ordinate patterns of gene expression by bio informatic data analysis using the model system of the present invention allows the identification of cell pathways and processes associated with apoptosis and survival. Following cluster analysis a cluster of genes were identified that contained several genes involved in neutrophil survival and whose transcription is unregulated upon GM-CSF treatment. This cluster is termed the survival cluster. Among these genes, one group that could be clearly identified are those genes whose protein products are involved in protecting the cell against an increased oxidative environment.

**Figure 17** on further analysis of the survival cluster we identified transcription of the biofunctional enzyme phosphofructokinase-2 (PFK-2)/ fructose 2,6-bisphosphatase (FBPase-2) which regulates the cellular concentration of fructose 2,6 bisphosphate and which in turn can regulate the function of phosphofructokinase-1.

**Figure 18** shows that ectopic expression of superoxide dismutase in Hela cells shows a dose-dependent resistance to apoptosis induced by cisplatin, confirming that superoxide has an anti-apoptotic function.

**Figure 19** shows that the ectopic expression of superoxide dismutase in HeLa cells confers resistance to apoptosis induced by expression of the transcription factor p53.

#### **Brief Description of the Tables**

- 5    **Table 1** shows a morphological determination of apoptosis. The table shows that although there is a small amount of apoptosis occurring two hours post isolation, there is a large increase in the number of cells undergoing spontaneous apoptosis between 6-8 hours, with close to 50% of the cells showing apoptotic morphology at 8 hours.
- 10   **Table 2** shows that an increase in superoxide production, as detected by flow cytometry occurs during the spontaneous apoptosis of neutrophils.

- 15   **Table 3** summarizes those genes regulated early in GM-CSF mediated inhibition of neutrophil apoptosis, as identified by Suppression Subtractive hybridisation (SSH). A sample of clones differentially expressed in these libraries are sequenced as described. A number of clones correspond to known genes, whilst many others are unique and do not have any identity with any expressed sequence in the public databases. The sequence identities are presented in table 3.

- 20   **Table 4** shows the GM-CSF mediated inhibition of neutrophil apoptosis is associated with significant changes in global mRNA expression. Table 4a. All 17 genes upregulated by GM-CSF at 2 hours are blocked by gliotoxin. Table 4b, of 76 genes upregulated by GM-CSF for 4 hours, 60 are blocked by gliotoxin. Table 4c, another 156 genes upregulated by GM-CSF at 2 hours, 57 are blocked by gliotoxin.

25

#### **Detailed description of the invention**

- The regulation of many genes presently known to be involved in apoptosis, including caspase genes, is presently being investigated as a route to the manipulation of apoptotic processes. Such attempts suffer from a fundamental flaw, in that because
- 30   many of these genes/proteins become involved in apoptosis after the cell has made a

commitment to the apoptotic pathway, regulation of these genes can only serve to delay the onset of apoptosis and not to prevent it. The present invention avoids this fundamental drawback and provides a method by which genes involved in the early stages of apoptosis, whose expression is modulated by GM-CSF levels in the  
5 extracellular environment, can be identified and isolated.

The control of apoptosis in neutrophils is useful in the treatment of a number of diseases, including asthma, COPD, posttraumatic acute respiratory distress syndrome (ARDS), systemic lupus erythematosus (SLE), Inflammatory Bowel disease (IBD),  
10 end-stage renal disease (uremia), Cardiopulmonary bypass, rheumatoid arthritis, cutaneous allergic (leukocytoclastic) vasculitis (CAV), cystic fibrosis (CF), severe congenital neutropenia (SCN), Endotoxin (ET)-induced liver failure, acute myelogenous leukaemia and neutropenia following radiation and chemotherapeutic treatments for cancer.

15

The present invention provides a model system for identifying a gene product/s which modulate the transistion of a cell between a non-apoptopic state and an apoptopic state, by exposing the cell to an inhibitor of GM-CSF mediated inhibition of apoptosis; and then exposing the cell to GM-CSF and then identifying gene products whose  
20 expression has been modulated as a result of the treatment.

The present invention seeks to overcome the problems encountered by previous attempts aimed at identifying those genes involved in apoptosis. Many previous genomics studies have looked at one parameter eg. Apoptosis. This has resulted in an  
25 unwieldy number of candidate genes. Other studies have described cluster analysis across multiple experiments. The model system of the present invention differs in that it permits focused, multiple and opposing events to be studied, and the genes/polypeptides involved in these multiple events to be identified and characterised. For example, Apoptosis vs survival vs the inhibition of survival may be studied.

30

### **General Techniques**

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art (e.g., in cell  
5 culture, molecular genetics, nucleic acid chemistry, hybridisation techniques and  
biochemistry). Standard techniques are used for molecular, genetic and biochemical  
methods (see generally, Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2d  
ed. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. and  
Ausubel *et al.*, Short Protocols in Molecular Biology (1999) 4<sup>th</sup> Ed, John Wiley &  
10 Sons, Inc. which are incorporated herein by reference), chemical methods,  
pharmaceutical formulations and delivery and treatment of patients.

### **Determination of expression levels**

15 As indicated above, a number of individual gene product types may be screened for in  
the present invention. These products include polypeptides and nucleic acids. The  
expression levels assessed may be absolute levels of production of a particular  
polypeptide or nucleic acid, or the levels of production of a derivative of any  
polypeptide or nucleic acid. For example, the invention may be configured to measure  
20 the level of expression of a particular mRNA splice variant, or the amount present of a  
phosphorylated derivative of a particular polypeptide.

Where it is desired to monitor the levels of expression of a known gene product,  
conventional assay techniques may be employed, including nucleic acid hybridisation  
25 studies and activity-based protein assays. Kits for the quantitation of nucleic acids and  
polypeptides are available commercially.

Where the gene product to be monitored is unknown, however, methods are employed  
which facilitate the identification of the gene product whose expression is to be  
30 measured. For example, where the gene product is a nucleic acid, arrays of  
oligonucleotide probes may be used as a basis for screening populations of mRNA  
derived from cells.

### Arrays

Gene Arrays of oligonucleotides specific to gene sequences archived in public domain  
5 databases, such as GenBank, are available commercially from a number of suppliers  
(such as Incyte Genomics). Examples of such commercial arrays are in the form of  
either nucleotides spotted onto a membrane filter (such a nitrocellulose), or a solid  
support (such as glass). Commercial Gene Arrays are used to profile the patters of  
gene expression that are associated with the process of apoptosis in neutrophils.

10

Gene Arrays are additionally constructed in-house, by spotting nucleotide sequences  
derived from cDNA clones generated from in-house libraries or from cDNA clones  
purchased commercially. Such arrays allow the expression profiling of proprietary  
and/or novel nucleotide sequences.

15

Many of the cDNA sequences or EST (expressed sequence tag) sequences deposited in  
the public domain databases are derived from a restricted set of tissue types, such as  
liver, brain and foetal tissue. The cloning of in-house cDNA libraries that are focused  
to specific cellular events, such as inhibition of apoptosis by GM-CSF offers the  
20 possibility to identify, clone and characterise novel genes that are associated with this  
process. Similarly, the cloning of in-house cDNA libraries that are focused to specific  
tissue types, such as the neutrophil, offers the possibility to identify, clone and  
characterise novel genes whose expression is restricted to this cell type. Libraries  
(cDNA) constructed using a physical subtraction, such as the ClonTech 'Select' SSH  
25 (suppression hybridisation) method, allow the selective cloning of genes whose  
expression is differentially regulated in the process or cell type being studied. Gene  
Array technology is combined with SSH cDNA libraries to identify false-positives and  
further focus on truly differentially expressed genes. Clones from each SSH library  
constructed are picked, cultured and archived as glycerol stocks. The cDNA inserts  
30 contained within individual plasmid clone are PCR amplified and spotted onto in-  
house arrays. Differential expression is confirmed using hybridisation with a  
radiolabelled probe generated from the mRNA used for each reciprocal subtractions.

Arrays of nucleic acids may be prepared by direct chemical synthesis of nucleic acid molecules. Chemical synthesis involves the synthesis of arrays of nucleic acids on a surface in a manner that places each distinct nucleic acid (e.g., unique nucleic acid  
5 sequence) at a discrete, predefined location in the array. The identity of each nucleic acid is determined by its spatial location in the array. These methods may be adapted from those described in U.S. Patent No. 5,143,854; WO90/15070 and WO92/10092; Fodor *et al.* (1991) *Science*, 251: 767; Dower and Fodor (1991) *Ann. Rep. Med. Chem.*, 26: 271.

10

In a preferred aspect of the invention, arrays of nucleic acids may be prepared by gridding of nucleic acid molecules. Oligonucleotides may be advantageously arrayed by robotic picking, since robotic techniques allow the most precise and condensed gridding of nucleic acid molecules; however, any technique, including manual techniques, which  
15 is suitable for locating molecules at discrete locations on a support, may be used.

The gridding may be regular, such that each colony is at a given distance from the next, or random. If molecules are spaced randomly, their density can be adjusted to statistically reduce or eliminate the probability of overlapping on the chosen support.

20

Apparatus for producing nucleic acid microarrays is available commercially, for example from Genetix and Genetic Microsystems. Moreover, pre-prepared arrays of nucleic acid molecules are available commercially, for example from Incyte Genomics Inc. (Human LifeGrid<sup>TM</sup>). Such arrays will comprise expressed sequence tags (ESTs)  
25 representative of most or all the genes expressed in a cell or organism, thus providing a platform for the screening of mRNA populations from multiple ROS-treated cells.

Samples for mRNA population analysis may be isolated and purified by any suitable mRNA production method; for example an RNA isolation kit is available from  
30 Stratagene.

## 2D PAGE

For the monitoring of unknown polypeptide gene products, separation techniques such as 2 dimensional gel electrophoresis are employed. 2D PAGE typically involves sample preparation, electrophoresis in a first dimension on an immobilised pH gradient, SDS-PAGE electrophoresis in a second dimension, and sample detection. Protocols for 2D PAGE are widely available in the art, for example at <http://www.expasy.ch/ch2d/protocols/>, the contents of which as of 16.12.1999 are incorporated herein by reference.

10

Samples for 2D PAGE may be prepared by conventional techniques. In the case of one of the preferred cells for use in the invention, HL60 (promyelocytic leukemia cells), a monolayer culture of a human promyelocytic leukemia cell line is grown in a suitable medium, such as RPMI 1640 containing 10% foetal calf serum (FCS), and treated with GM-CSF as necessary. The suspension is transferred into a tube and the cells are centrifuged at 1000 g for 5 minutes. Supernatant is discarded and the cells are washed with RPMI 1640 without FCS. After centrifugation and removal of RPMI 1640,  $0.8 \times 10^6$  cells are mixed and solubilised with 60  $\mu$ l of a solution containing urea (8 M), CHAPS (4% w/v), Tris (40 mM), DTE (65 mM) and a trace of bromophenol blue. The whole final diluted HL60 sample is loaded on the first dimensional separation.

20

The method of the present invention advantageously employs a step of establishing a reference expression level for the gene products being investigated. This can be carried out before addition of GM-CSF to the cells, and serve as a standard for one or more subsequent assays; or it may be an integral part of every assay. For example mRNA or polypeptide populations from GM-CSF treated and untreated cells may be assessed simultaneously on a nucleic acid array or by 2D PAGE, and changes in expression patterns identified by direct comparison.

25

30

Analysis of 2D PAGE results, using appropriate software where necessary, reveals Polypeptides of interest may be isolated, sequenced and used to identify genes encoding them.

## 5 **General Applications of the Invention**

The model systems or discovery assays described herein provides the means to characterise the molecular mechanisms of apoptosis. In particular, these molecular mechanisms include the GM-CSF mediated inhibition of apoptosis. In addition, the strategy provides the means to identify, characterise, and clone molecules, including  
10 oligonucleotides and polypeptides, associated, both causally and consequentially, to the GM-CSF mediated inhibition of apoptosis.

We have shown that gliotoxin inhibits the GM-CSF mediated inhibition of apoptosis. By using an inhibitor of GM-CSF in a model of the GM-CSF mediated inhibition of  
15 apoptosis, the genes and gene products involved in multiple events may be identified and characterised.

In a preferred configuration of the present invention, cells such as primary human neutrophils are isolated and purified from the peripheral blood of individuals. Upon  
20 culture in a serum-containing cell culture medium these neutrophils undergo 'spontaneous apoptosis' (Haslett, Clinical Science 83, pp639-648, 1992). Apoptosis as measured by morphology is apparent and increases from approximately 6 to 8 hours following isolation of the cells. The onset of apoptosis is further characterised using additional markers for apoptosis, such as caspase activation and cell shrinkage. These  
25 assays serve to identify the earliest measurable onset of the cells commitment to apoptosis. Intracellular events which drive this commitment of the cell to apoptosis occur before and around this earliest measurement of the commitment.

In a further preferred configuration of the present invention, cells such as HL60 or  
30 HeLa, are cultured and treated with external agents that induce apoptosis through the endogenous production of ROS, such as UV-irradiation, cytokines such as TNF $\alpha$ , or chemotherapeutic agents such as cisplatin. Upon treatment of cells with UV-



- irradiation,  $\text{TNF}\alpha$ , or cisplatin, apoptosis is induced; this apoptosis has been shown to be mediated by an endogenous production of ROS (Miyajima A, Nakashima J, Yoshioka K, Tachibana M, Tazaki H, Murai M (1997) Role of reactive oxygen species in cis-dichlorodiammineplatinum-induced cytotoxicity on bladder cancer cells.
- 5 Br J Cancer 76 (2) pp206-10; Chan, W-H, Yu, J-S (2000) Inhibition of UV Irradiation-induced oxidative stress and apoptotic biochemical changes in human epidermal carcinoma A431 cells by genestein. J. Cell Biochem. 78 pp73-84; Sidoti-de Fraisse C, Rincheval V, Risler Y, Mignotte B, Vayssiere JL (1998)  $\text{TNF-}\alpha$  activates at least two apoptotic signalling cascades. Oncogene (13): pp1639-51).
- 10 Apoptosis as measured by morphology is apparent as determined by DNA fragmentation. The onset of apoptosis is characterised using markers for apoptosis. These assays serve to identify the earliest measurable onset of the cells commitment to apoptosis. Intracellular events which drive this commitment of the cell to apoptosis occur before and around this earliest measurement of the commitment.
- 15 The characterisation of the apoptosis process and GM-CSF involvement in this model system permit the key early intracellular events which control the GM-CSF mediated inhibition of apoptosis, the molecular signalling pathways mediating or associated with GM-CSF mediated inhibition of apoptosis.
- 20 We have shown that gliotoxin inhibits the GM-CSF mediated inhibition of apoptosis and that the effect is mediated at least in part via  $\text{NF}\kappa\text{B}$  and/or  $\text{NAD(P)H}$ . In addition, the model system has been used to show that the protective effect of GM-CSF is mediated, at least in part, via tyrosine phosphorylation.
- 25 Global gene expression patterns allows a detailed characterisation of the mechanisms underlying and causal to the apoptotic process. Indeed we show GM-CSF mediated inhibition of apoptosis involves an increase in intracellular tyrosine phosphorylation in neutrophils. In addition, we show that genes regulated early in GM-CSF mediated
- 30 inhibition of neutrophil apoptosis include superoxide dismutase (Mn-SOD), and Hypoxia Inducible factor (HIF $\alpha$ ). A number of other genes were identified whose gene products are involved in apoptosis and survival including those whose protein

products are involved in protecting the cell against an increased oxidative environment. These include catalase and ferritin as well as superoxide dismutase. Thus, we conclude that GM-CSF protects the cell by reducing the harmful effects of reactive oxygen species.

5

In addition, phosphofructokinase-2 (PFK-2)/fructose 2,6-biphosphatase (FBP ase-2) transcription is increased. PFK-2 can regulate the cellular concentration of fructose 2,6 biphosphate, and in turn the activity of phosphofructokinase-1. GM-CSF also causes up-regulation of fructose 1,6 biphosphatase and down regulation of  
10 phosphofructokinase 1. These enzymes are components of the pentose-phosphate pathway. From these studies it could be concluded that GM-CSF increases the formation of glucose 6 phosphate which can be utilised in reducing NADPH via the pentose phosphate pathway.

15 Analysis of global gene expression patterns, using for example 'cluster analysis' to group genes with similar temporal patterns of expression, allows a detailed characterisation of signalling pathways and cellular processes associated with treatments, as detailed above. Indeed we show that this analysis, of gene expression using our model system, identifies genes that are 'known' to be co-regulated and co-  
20 expressed, and which belong to the same signalling pathways or cellular process. We also show that many genes (the expression patterns of which are grouped, or clustered, with those genes that have know function) identified and cloned have little or no know known biological function, or which have no existing sequence homology to previously cloned genes. These genes may represent the most attractive therapeutic  
25 targets.

The invention provides the means to allow the identification and isolation of genes or polypeptides that may represent valuable and attractive therapeutic targets for the control of apoptosis in a range of conditions and diseases.

30

**Example 1.**

Establishment and characterisation of a 'Model Cell-System / Discovery Assay' to  
5 study the molecular mechanisms of cellular response to GM-CSF mediated inhibition  
of apoptosis in primary human neutrophils.

This example describes the establishment of a model cell system in primary human  
neutrophils. The isolation and culture of primary human neutrophils is described.  
10 Neutrophils are allowed to undergo spontaneous apoptosis in culture. This spontaneous  
apoptosis is accompanied and characterised by the endogenous production of ROS.  
This spontaneous apoptosis is inhibited by the addition of GM-CSF. This 'Model Cell  
System' forms the basis of a novel 'Discovery Assay' that is then used for the  
characterisation, identification and functional validation of nucleotide (genes, mRNAs)  
15 or polypeptide (proteins, peptides) sequences associated with and potentially  
responsible for the molecular mechanisms of apoptosis, including; 1) the cells attempt  
to combat apoptosis in the cell, 2) the cellular signaling pathways, 3) the initiation of  
the apoptosis process, and 4) are associated with the processes of apoptosis and  
defence that will aid in the understanding of key pathways, processes and  
20 mechanisms.

We demonstrate the utility of this model system to 'discover' early regulated genes  
associated with GM-CSF inhibition of apoptosis, by the discovery of genes that have  
'known' involvement in cell survival. Similarly we demonstrate the identification of  
25 genes regulated 'early' in GM-CSF inhibition of apoptosis that have no known  
biological function i.e. ESTs from both commercial filters and from our own libraries  
of differentially expressed genes isolated by Suppression Subtractive Hybridisation  
(SSH). By association with known genes, as described, these novel genes themselves  
are identified as candidate apoptosis genes that may similarly function in 1) the cells  
30 attempt to combat apoptosis in the cell, 2) the cellular signaling pathways, 3) the  
initiation of the apoptosis process, and 4) are associated with the processes of

apoptosis and defence that will aid in the understanding of key pathways, processes and mechanisms.

### **Isolation and culture of primary human neutrophils**

5

Whole blood (20–50 ml) is taken from normal healthy volunteers by venepuncture. Coagulation is prevented by the use of sodium citrate. A 6% dextran (mol wt 509,000; Sigma) saline solution is added in 1:4 ratio to whole blood and the erythrocytes allowed to sediment for 45 minutes at 22°C. The buffy coat is then under-layered with  
10 5 ml Ficoll-Paque (Pharmacia LKB Biotechnology) and centrifuged (300g, 30 min) to pellet granulocytes and erythrocytes (Boyum, 1968). The pellet is resuspended in 1 ml cell culture tested water (Sigma) for 40 sec., followed by the addition of 14ml Hanks buffer (Sigma) and centrifuged (300g, 10 min.). This lysis step is repeated to ensure removal of all erythrocytes. The remaining pellet is resuspended in RPMI 1640  
15 supplemented with 10% foetal calf serum (Sigma), L-glutamine (2mM), penicillin (100 U/ml; Sigma), streptomycin (100 µg/ml; Sigma) and amphotericin B (2.5 µg/ml; Sigma). Cell number and viability is checked using trypan blue exclusion (Boyum, (1968) *Scand J Clin Lab Invest Suppl*; 97:77-89).

20 Isolated neutrophils are maintained at a density of  $2 \times 10^6$ / ml in RPMI 1640 supplemented with 10% foetal calf serum (Sigma). Further additions to the medium included L-glutamine (2mM), penicillin (100 U/ml), streptomycin (100 µg/ml) and amphotericin B (2.5 µg/ml) (Sigma). Cells are incubated at 37°C in a humidified CO<sub>2</sub> (5%) incubator.

25

### **Cellular/biochemical characterisation of apoptosis**

A range of assays are established and used to measure the magnitude and temporal induction of apoptosis. The earliest biochemical measurement of the apoptosis  
30 phenotype by these assays is considered to be the point beyond which the cells are 'Committed' to the process of apoptosis. In addition, these measurements determine the reproducibility of induction of apoptosis in the model systems. Furthermore, these

measurements determine the cellular mechanisms of apoptosis in these systems (such as whether apoptosis is caspase-dependent or caspase-independent).

Primary human neutrophils are isolated and purified from the peripheral blood of normal healthy individuals. Upon culture in a serum-containing cell culture medium these neutrophils undergo 'spontaneous apoptosis' (Haslett, Clinical Science 83, pp639-648, 1992).

Our data indicate the earliest detection of neutrophil spontaneous apoptosis, and thus a time of 'commitment to die' at 6 to 8 hours post-isolation.

#### **Caspase activation assay**

Caspase activity (Caspase-3) is measured using a commercial kit (CaspACE™ Assay System, Promega). The methodology is essentially as described by the manufacturer. Cells are removed from culture and centrifuged (300g/ 10 min) at 4°C. The pellet is kept on ice, washed in ice-cold Hanks buffer and then resuspended in Cell Lysis Buffer at  $10^8$ / ml. Cells are lysed by freeze-thaw once, incubated on ice for 15 min, followed by centrifugation (15,000g /20 min) at 4°C. The caspase 3 activity present in the supernatant fraction is measured using the absorbance at 405nm and normalised per mg of protein in the supernatant. Figure 2 represents the increase in caspase activity during the incubation period. While caspase activity is observed at the earlier time points, there is a dramatic increase between 8 and 20h of culture, which is in agreement with our morphological data.

25

#### **Morphological Determination of Apoptosis**

A cell aliquot (100µl) is removed from culture and using an IEC Centra-7 centrifuge equipped with a Cytobucket™ adapter, a monolayer of cells is concentrated onto standard microscopic slides. Preparations are allowed to air dry prior to fixing in Rapi-Diff (Diagnostic Developments, UK) solution A (reactive ingredient 100% methanol). Slides are air dried prior to immersion in solution B containing; eosin Y

30

(0.1% w/v), formaldehyde (0.1% w/v), sodium phosphate dibasic (0.4% w/v) and potassium phosphate monobasic (0.5% w/v). Excess stain is drained from the slide prior to immersion in solution C, containing methylene blue (0.4%w/v), Azure A (0.04% w/v), sodium phosphate dibasic (0.4% w/v), potassium phosphate monobasic (0.05% w/v) and potassium phosphate monobasic (0.4% w/v), to counterstain the cytoplasm. Excess dye is rinsed; the slides air-dried and mounted in DPX aqueous mountant (BDH Laboratory Supplies, U.K.). Morphological examination is then carried out by light microscopy for the presence of apoptotic cells as determined by the loss of membrane asymmetry and condensation of cytoplasm and nuclei (Cotter and Martin, 1996).

Apoptosis as measured by morphology is apparent and increased from approximately 6 hours following isolation of the cells. Our results demonstrate that although there is a small amount of apoptosis occurring at 2h post isolation, there is a large increase in the number of cells undergoing spontaneous apoptosis between 6 -8h resulting in close to 50% of the cells showing apoptotic morphology at 8 hours (See Table 1 and Figure 3).

#### Cell Shrinkage Determination of Apoptosis

Onset of apoptosis as determined by cell shrinkage is determined by flow cytometry. Isolated cells are set up in culture at a concentration of  $2 \times 10^6$ /ml. At the indicated time points a sample is removed and the forward and side scatter parameters of the cells are measured by flow cytometry using a Becton Dickinson FACScan equipped with CellQuest software. Cell shrinkage is detected by a reduction in forward scatter parameters. Determination of apoptosis inhibition is calculated by the percentage of cells with reduced forward scatter parameters, in untreated cultures, minus percentage of cells with reduced forward scatter parameters in treated cultures.

Our results for spontaneous neutrophil apoptosis demonstrate that although there is a small amount of apoptosis occurring at 2h and 4h post isolation, there is a large increase in the number of cells undergoing spontaneous apoptosis from 6h with close to 50% of the cells showing apoptotic morphology at 6 hours (See Figure 3).

**Neutrophil spontaneous apoptosis is accompanied by the endogenous production of ROS, in the form of  $O_2^-$  and  $H_2O_2$**

5

Human neutrophils are specialised for the phagocytosis and lysis of bacteria. This lysis is accompanied by an enzyme complex called NADPH oxidase (Babior, B.M. (1978)). Oxygen dependent microbial killing by phagocytes. New England Journal of Medicine, 298(12) pp659-668. NADPH oxidase is assembled at the membrane of the  
10 phagosome and generates ROS, in the form of superoxide ( $O_2^-$ ). This endogenous production of ROS contributes to the bacterial lysis. NADPH oxidase activity is present in normal peripheral blood neutrophils and is further increased by cellular activation. Consequently, neutrophils have the capacity to generate significant amounts of endogenous ROS. As with other cell types, ROS in the form of superoxide may also  
15 be produced by mitochondria during oxidative respiration.

Chronic Granulomatous Disease (CGD) is characterised by a molecular defect and loss of activity of the neutrophil NADPH Oxidase. Notably, neutrophils isolated from patients with CGD have a significantly delayed spontaneous apoptosis Kasahara Y,  
20 Iwai K, Yachie A., Ohta, K., Konno A., Seki H., Miyawaki, T and Taniguchi N. (1997) Involvement of reactive oxygen intermediates in spontaneous and CD95(Fas/Apo-1)- mediated apoptosis of neutrophils. Blood 89 (5) pp1748-1753.

Increased  $H_2O_2$  occurs upon neutrophil activation, with. e.g. PMA, and this is  
25 associated with increased rate of apoptosis. (Lundqvist-Gustafsson H, and Bengtsson T. (1999) Activation of the granule pool of the NADPH oxidase accelerates apoptosis in human neutrophils. J. Leuk. Biol. 65: pp196- 204).

Our results demonstrate that we can detect an increase in the superoxide production, as  
30 detected by flow cytometry (See Table 2). The greatest number of cells producing superoxide anions peaked 3-4 hours post isolation, before returning to background levels.

Our results also demonstrate that this peak in  $O_2^-$  production precedes an increase in intracellular  $H_2O_2$  production (See Figure 4). This temporal pattern is consistent with the  $H_2O_2$  production resulting from the action of superoxide dismutase, an enzyme  
5 present in neutrophils and which catalyses the conversion of  $O_2^-$  to  $H_2O_2$ .

#### **Dose responsiveness of the anti-apoptotic effect of GM-CSF.**

Primary human neutrophils are isolated and purified from peripheral blood of normal  
10 healthy individuals. Neutrophils are resuspended in serum containing culture medium together with various amounts of GM-CSF at a density of  $2 \times 10^6$ /ml, with 100 $\mu$ l plated into a 96 well plate and cultured for 18h at 37°C. After this time 10 $\mu$ l of MTT (5mg/ml) is added to the cultures and incubated for a further 4h at 37°C before solubilisation of the purple coloured formazan with acidic isopropanol. Optical  
15 densities are read at 570nm using a plate reader. Our results demonstrate a direct correlation between survival and concentrations of GM-CSF added to the culture medium (Figure 5).

#### **Determination of the temporal relationship between GM-CSF addition and 20 survival**

A time course of GM-CSF additions is carried out to neutrophils in order to determine up to what stage GM-CSF can induce survival in neutrophils. Primary human neutrophils are isolated and purified from peripheral blood of normal healthy  
25 individuals. Neutrophils are resuspended in serum containing culture medium at a concentration of  $2 \times 10^6$ /ml, with 100 $\mu$ l/well plated into a 96 well plate and culture at 37°C commenced. At the indicated time points GM-CSF (50U/ml) is added to the neutrophils and culture continued until 20h post initiation of culture. After this time, 10 $\mu$ l of MTT (5mg/ml) is added to the cultures and incubated for a further 4h at 37°C  
30 before solubilisation of the purple coloured formazan with acidic isopropanol. Optical densities are read at 570nm using a plate reader. Our results demonstrate that GM-CSF



can be added to neutrophils as late as 5 hours post isolation and not get any discernible loss in the protective effect of this cytokine. However, if administration of the cytokine is delayed for 20h post isolation, the protective effect is zero in 24h cultures. This data indicates that the commitment to die for the vast majority of neutrophils is later than 5 hours post isolation (See Figure 6). This result is in agreement with our other findings that morphological characteristics of apoptosis occurs between 6 and 8 hours.

#### **Determination of GM-CSF "on time" sufficient for apoptosis delay**

10

We performed timed additions of anti-GM-CSF antibody to neutrophils in order to determine how immediate the anti-apoptotic effects induced by GM-CSF occur. Primary human neutrophils are isolated and purified from peripheral blood of normal healthy individuals. Neutrophils are resuspended in serum containing culture medium containing 5 U/ml of GM-CSF at a concentration of  $2 \times 10^6$ /ml, with 100  $\mu$ l/well plated into a 96 well plate and culture at 37°C commenced. At the indicated time points additions 10  $\mu$ g/ml anti-GM-CSF are made to the neutrophils and cultured for 18h at 37°C. After this time, 10  $\mu$ l of MTT (5mg/ml) are added to the cultures and incubated for a further 4h at 37°C before solubilisation of the purple coloured formazan with acidic isopropanol. Optical densities are read at 570nm using a plate reader. Comparable survival is obtained when addition of anti- GM-CSF is delayed for 3 hours following isolation as to when no neutralising antibody is added (See Figure 7). In contrast, when neutralising antibody is added before 3 hours, survival is directly linked to the addition time. This indicates that the survival mediated by GM-CSF is not immediate and that cells must be exposed to the cytokine for a defined period of time ("on time") before survival is conferred.

30

## Example 2

### **Intracellular Tyrosine Phosphorylation is required for GM-CSF inhibition of neutrophil apoptosis.**

This example characterises the role that intracellular phosphorylation plays in delaying the apoptotic process of neutrophils. As can be seen in Fig 8, the addition of low doses of the phosphatase inhibitor Phenylarsine Oxide (PAO) to neutrophils increase the survival of the cells after 24h in culture. Therefore it can be concluded that intracellular tyrosine phosphorylation plays a pivotal role in the protective effects on anti-apoptotic agents described in this study.

Primary human neutrophils are isolated and purified from peripheral blood of normal healthy individuals. Neutrophils are resuspended in serum containing culture medium at a concentration of  $2 \times 10^6/\text{ml}$  and  $100\mu\text{l}$  aliquots plated into a 96 well plate. The protein phosphatase inhibitor Phenylarsine Oxide (PAO) is added to select wells at time 0h, at various concentrations ( $0.1 - 1\mu\text{M}$ ) and culture at  $37^\circ\text{C}$  commenced. After the indicated time,  $10\mu\text{l}$  of MTT ( $5\text{mg}/\text{ml}$ ) are added to the cultures and incubated for a further 4h at  $37^\circ\text{C}$  before solubilisation of the purple coloured formazan with acidic isopropanol. Optical densities are read at  $570\text{nm}$  using a plate reader.

## Example 3

### **Fungal metabolite Gliotoxin blocks GM-CSF inhibition of neutrophil apoptosis.**

This example describes the identification of an inhibitor for the GM-CSF mediated inhibition of neutrophil apoptosis. The use of this inhibitor allows us to focus in on the specific biochemical events mediating the GM-CSF survival events. In turn one is able to remove some of the noise associated GM-CSF treatment.

Primary human neutrophils are isolated and purified from peripheral blood of normal healthy individuals. Neutrophils are resuspended in serum containing culture medium containing  $5 \text{ U}/\text{ml}$  of GM-CSF at a concentration of  $2 \times 10^6/\text{ml}$ . Also added to the

culture mix is either 0.1µg/ml of the fungal metabolite Gliotoxin (a specific inhibitor of NFκB) or its inactive analogue bis -Dethio -bis (Methylthio) Gliotoxin, with 100µl/well plated into a 96 well plate and culture at 37°C commenced. After the indicated time, 10µl of MTT (5mg/ml) are added to the cultures and incubated for a  
5 further 4h at 37°C before solubilisation of the purple coloured formazan with acidic isopropanol. Optical densities are read at 570nm using a plate reader.

Figure 9 demonstrates that gliotoxin effectively blocks the GM-CSF inhibition of neutrophil apoptosis. This blocking effect is not seen when the inactive analogue of  
10 gliotoxin, methylgliotoxin is added with GM-CSF. No increased neutrophil apoptosis is seen with the addition of gliotoxin alone to isolated neutrophils demonstrating that the effect is specific to and limited to a reversal of the protective effects of GM-CSF.

Gliotoxin has been shown to inhibit the transcription factor NFκB (Ward et al. 1999,  
15 J. Biol. Chem. 274. 4309-4318). In turn Yoshida et al have identified an alternative mode of action of gliotoxin. These investigators demonstrated that gliotoxin inhibited NADPH oxidase and consequently prevented the onset of superoxide generation by human neutrophils in response to phorbol myristate. (Yoshida et al Biochem Biophys Res Commun 2000, 268(3) 716-23).

20

By comparing GM-CSF treated, but specifically inhibited for NFκB (and/or NADPH oxidase) with GM-CSF treated alone, allows us to identify specific genes that fit the criteria of a) transcribed in the presence of GM-CSF b) and whose transcription is mediated via a specific transcriptional factor (and/or signal transduction pathway).  
25 Our data demonstrates that NFκB binding to DNA (and/or NADPH oxidase activity) is a prerequisite for GM-CSF mediated survival (Fig 9).

#### Example 4

30

**Characterisation and discovery of oligo/polynucleotides 'Genomics' associated with GM-CSF inhibition of neutrophil apoptosis.**

This example describes the characterization, cloning and analysis of  
5 oligonucleotide/polynucleotide sequences whose expression changes are associated with GM-CSF inhibition of neutrophil apoptosis.

In one example, Suppression Subtractive Hybridisation is used to identify and clone  
cDNA sequences derived from differentially expressed genes, associated with  
10 neutrophil apoptosis, GM-CSF inhibition of neutrophil apoptosis, and the inhibition of this effect using the fungal metabolite Gliotoxin. Such differential gene expression is associated with a measurable apoptotic phenotype.

In another example, commercial microarrays are used to measure global gene  
15 expression associated with GM-CSF inhibition of neutrophil apoptosis, and the inhibition of this effect using the fungal metabolite Gliotoxin.. Analysis of such microarray results identifies genes whose expression pattern changes (either up-regulation or down-regulation) in an association with a measurable apoptotic phenotype.

20

**Suppression Subtractive Hybridisation (SSH) cloning of differentially expressed genes**

This example describes the process of Suppression Subtractive Hybridisation (SSH).  
25 SSH is an open' differential cloning system. Unlike microarray, which requires the analysis of known gene sequences, SSH has the potential to clone all mRNAs that are differentially expressed between a control and test population. We also describe substantial modification to the commercial SSH procedure that enhances its performance.

30

### Total RNA isolation

Primary or cultured cells are split into control and treatment groups and the treatment group is then challenged with apoptotic stimuli or inhibitors as appropriate. Total RNA is then prepared from both groups using acid phenol/guanidine isothiocyanate extraction (RNAzol B; Biogenesis), or RNeasy RNA preparation kits (Qiagen). Any contaminating genomic DNA is removed by DNase treatment (DNase I, Gibco-BRL). In this example, RNA is prepared from neutrophil cells following treatment with GM-CSF (50units/ml), Gliotoxin (10 $\mu$ M) or MethylGliotoxin (10 $\mu$ M). RNA is also prepared from neutrophils that have not been exposed to drug (i.e. as an untreated control). RNA is prepared from these cells using two sequential extractions with RNAzol B.

### Extraction of mRNA

Total RNA prepared as described above is used to prepare mRNA, using the Oligotex mRNA purification kit (Qiagen), or a similar system (Clontech or Stratagene). Briefly, mRNA is purified by passing the total RNA over an oligo-dT column. The oligo-dT may be attached to cellulose or glass beads or biotin, and the column may be either spin or gravity format. The bound mRNA is subsequently washed and eluted, ready for use in subtractive hybridisation.

### Preparation of SMART cDNA

When the quantity of total RNA is in limiting amounts, SMART cDNA amplification can be used to prepare template for the SSH reaction. SMART cDNA synthesis can start with either total or poly-A<sup>+</sup> RNA and depends upon a feature of the reverse transcriptase enzyme which causes the addition of unmatched deoxycytidines to the 3'-end of the cDNA. An oligonucleotide which has an oligo(dG) sequence at its 3'end pairs with the deoxycytidine stretch, creating an extended template. The reverse transcriptase can then switch strands and continue replicating to the end of the oligonucleotide. The resulting cDNA contains a primer site at each end, which can be used for PCR. The application of limited rounds of amplification then conserves the

relative abundance of each cDNA, while significantly increasing the amount of material available for subsequent manipulation.

In this example, SMART cDNA amplification kits are obtained from Clontech.

5 SMART cDNA is prepared using 1µg of total RNA from each time point. The reverse transcribed material is amplified to 17 cycles (to maintain a linear amplification), following which the cDNA is size fractionated over a Nucleospin chromatography column. SSH is performed on mRNA purified from treatment or control cells essentially as described (Diatchenko *et al* 1996), using a PCR-select cDNA subtraction

10 kit (Clontech, K1804). Briefly, cDNA is synthesised according to the SMART protocol for two treatment groups (driver and tester respectively). The resulting cDNA is digested with a restriction enzyme generating a blunt end product (in this example *RsaI*). The tester cDNA is divided into two subsets and distinct adaptor molecules are ligated to each of the cDNA pools. These two samples of tester cDNA are then

15 separately combined with driver cDNA in a solution containing 50mM HEPES, pH 8.3; 0.5M NaCl; 0.02mM EDTA, pH 8.0. Following denaturation, (1.5min, 98°C), the tester and driver cDNAs are allowed to anneal for 10hrs at 68°C. After this first hybridisation, the two samples are combined and a fresh portion of heat denatured driver cDNA is added. The samples are allowed to anneal for a further 10 hours at

20 68°C. The hybridised cDNA is then diluted in a solution containing 20mM HEPES, pH 8.3; 50mM NaCl; 0.2mM EDTA) and heated at 72°C for 7 minutes, prior to PCR amplification. PCR is performed under standard conditions using the Advantage cDNA PCR kit (Clontech). Only cDNAs that have the correct primer combination, the differentially expressed cDNAs, will amplify exponentially. Reciprocal subtractions

25 are prepared for each pair of samples, which thereby allows identification of both up and down-regulated genes.

#### Construction of SSH cDNA libraries

30 PCR products from the subtractive hybridisation are inserted into a TA cloning vector, in this example the pAdvanTage cloning kit (Clontech). This library of differentially

expressed cDNAs is then transformed to *E.coli* and the transformants selected for plasmid DNA prep and sequence analysis.

#### **Plasmid miniprep**

5

This example describes the extraction of double stranded plasmid DNA from cDNA clones.

Individual colonies (*E. coli* DH5 $\alpha$  or TOP10F') are picked into 96-deep well culture  
10 blocks containing 2.3 ml LB + ampicillin. The culture blocks are shaken at 300 rpm  
37°C for 24 h. Plasmid DNA is isolated using MultiScreen-FB and MultiScreen-NA  
96-well plates (Millipore). The methodology is as described by the manufacturer.

#### **DNA sequencing**

15

This example describes the di-deoxy sequencing of cloned cDNA inserts within the vector pT-AdvanTAge (Clontech).

Plasmid miniprep DNA (100 ng to 5  $\mu$ g) is sent to MWG Biotech for contract  
20 sequencing. Sequencing reactions are primed using one of the following universal  
primer sequences:

M13 (-24) Reverse Primer: 5' aac agc tat gac cat g 3'

M13 (-48) Reverse Primer: 5' agc gga taa caa ttg cac aca gga 3'

25 M13 (-20) Forward Primer: 5' gta aaa cga cgg cca gt 3'

M13 (-40) Forward Primer: 5' gtt ttc cca gtc acg ac 3'

T3 Primer: 5' aat taa ccc tca cta aag gg 3'

T7 Primer: 5' gta ata cga ctc act ata ggg c 3'

30 **Use of microarray to confirm differential expression of SSH clones**

The SSH procedure for the cloning of differentially expressed gene products also generates a portion of artifactual cDNAs 'false positives' which are not differentially expressed. This example describes our modifications to the commercial SSH, by the use of microarray to confirm the identity of truly differentially expressed clones prior to the laborious task of sequencing.

Approximately 1000 colonies are isolated from each reciprocal SSH cDNA library. cDNA insert sequences are amplified by PCR and spotted onto microarray filters as described above. Duplicate filters are hybridised with radio-labeled cDNA probes generated from the reciprocal RNA material used to generate each SSH library (i.e. if an SSH library is made from a subtraction between diseased vs. normal cells, then one filter is hybridised with probe synthesised from RNA isolated from diseased cells and the other from normal cells) i.e. 'first round of screening'. An analysis of the filters identifies which cDNA clones are differentially expressed (See Figure 10).

Positive clones from the first round of screening are subsequently submitted to GeneScreen (Dorset, UK) for preparation of a micro array. This array is then hybridised against radiolabelled probes of choice to further analyse the expression profiles of the mRNAs corresponding to the isolated DNA sequences (See Figure 11).

These arrays may be additionally analysed using the ArrayVision software as described above. The output from this program may be normalised and further analysed using the Cluster and Treeview software, as described above.

## **25 Measurement of global gene expression by 'Microarray'**

This example describes the process of microarraying (in the context of both a commercial filter based microarray, or commercial spotted filter microarrays (as described above)) and its use to profile gene expression of thousands of genes simultaneously. The microarray process can be separated into three parts: the filter, the hybridisation of radiolabelled cDNA probes, and the detection and quantitation of the microarray results.



### **The microarray filter**

This example describes the use of the Human LifeGrid<sup>TM</sup> microarray filters obtained  
5 from Incyte Genomics (USA). These filters contain cDNA probes representing  
approximately 8,400 human mRNAs. This example also describes the use of filter  
microarrays spotted commercially by GeneScreen (Dorset, UK).

### **10 Synthesis of labelled probes**

This example describes the synthesis of a radiolabelled cDNA from total cellular  
mRNA. The labeled cDNA is used to 'probe' DNA fragments, which have been  
immobilised on to a filter membrane, by complementary hybridisation.

15

Methodology is as described by manufacturer, for Human LifeGrid<sup>TM</sup> arrays.  
Essentially, total cellular RNA (1 µg to 20 µg) or polyA<sup>+</sup> mRNA (100 ng to 5 µg) is  
incubated with an oligo (dT) primer. Primed RNA is reverse transcribed to first stand  
cDNA in a reaction containing M-MLV reverse transcriptase (RT; alternatively  
20 Superscript II is used (Life Sciences)), RT buffer, dNTPs and [ $\alpha$ -<sup>33</sup>P] dCTP (2000-  
4000 Ci/mmol) at 42°C for 1 to 5 hours. Unincorporated nucleotides are removed using  
spin-columns and the labeled probe stored at -80°C until required.

Labeled probes may also be generated from cDNA, genomic DNA or PCR products.  
25 In each case a random primed labeling procedure can be used, for example the Ready-  
Prime Labeling kit (APBiotech), applied as per manufacturers instructions.

### **Hybridisation of filter based microarrays**

30

This example describes the complementary hybridisation of radiolabelled cDNA probe to DNA fragments immobilised onto a membrane (typically a nylon or nitrocellulase filter).

- 5 Methodology is as described by manufacturer, for Human LifeGrid™ arrays. Essentially, membrane filters are pre-hybridised in hybridisation buffer (5 to 20 ml) at 42°C for 2 to 16 h using a hybridisation oven (Hybaid). Following pre-hybridisation, the labeled cDNA probe is added to fresh hybridisation buffer (5 to 20 ml) and hybridised at 42°C for 14 to 16 h. Following hybridisation, the hybridisation mix is  
10 removed and the filters washed with 2 x SSC buffer at RT for 5 min., twice with 2 x SSC, 1% SDS buffer at 68°C for 30 min. and twice with 0.6 x SSC, 1% SDS buffer at 68°C for 30 min.

#### **Quantitative imaging and analysis of microarray filters**

15

- This example describes the use of a STORM Phosphoimager to quantitatively image positive signals across the filter arrays. Hybridised filters are wrapped in plastic wrap (Saran) and exposed to a Low-Energy Phosphoimaging screen (Molecular Dynamics). The screen is then placed on the phosphoimager and the gel image captured by  
20 scanning at a resolution of 50 microns (See Figure 12).

- The captured image file is then analysed using software such as Array Vision (Imaging Research Inc.; See Figure 13). In this example we implement analysis with ArrayVision v5.1. This program contains facilities for spot detection and  
25 quantification, and background detection and quantification. This data is then exported to a text file for further analysis. A variety of data fields are exported from the ArrayVision analysis, including; Spot Label, Position, Density, Background, and particularly, Background subtracted density (sDens) and signal/noise ratio (S/N). In this example, the exported text file is up-loaded to an SQL-7.0 database, to populate a  
30 table containing array data from all experiments. As the data is imported to the database, a Normalisation factor is calculated and the sDENS values modified accordingly. This Normalised data is stored in a newly created column within the

table. The Normalisation factor facilitates accurate comparison between datasets. A number of different calculations may be used. A normalization factor may be derived from Linear Regression calculated by reference to housekeeping genes. Alternatively, the Global Mean is calculated as the average of the sDens values across all of the arrays to be compared and a normalisation factor is then derived by division of the overall spot density with the Global Mean value. Spot density values (individual sDens) are then corrected by multiplying across all values with the normalisation factor. In a similar approach a Global Geometric Mean normalization factor may be calculated and used to adjust the dataset. The data from multiple hybridisation experiments can then be stored in a suitable format, for example in an Access or SQL 7.0 database.

Comparison between arrays generates an output file containing the gene identifier and the fold-change in expression relative to the reference dataset. Fold change, ( $T_x$  vs  $T_y$ ), is calculated by dividing the normalised spot density values of  $T_x$  with  $T_y$ . In this example, multiple time-course experiments are prepared and fold-change values calculated with reference to the T0 time point.

The fold change data derived from comparison of multiple hybridisation experiments can be analysed using a variety of approaches, including hierarchical clustering, (supervised or unsupervised), k-means clustering or self-organising maps. Software enabling these analyses includes the Cluster and Treeview software (M.Eisen, Stanford Uni, USA), J-Express (European Bioinformatics Institute), GeneMaths (Applied Maths, Belgium) or GeneSpring (Silicon Genetics, USA). In this example hierarchical clustering is implemented using the GeneMaths software. Trees are generated using the UPGMA algorithm with distance calculated using the Pearson similarity metric. Alternatively Euclidean distance metrics are used.

#### **Simplification of Fold-change data**

Following cluster analysis, fold-change data can be difficult to interpret owing to either a very large dataset and/or a wide range in fold change values. The visualization and interpretation of these datasets may be simplified using codes or combined codes.

In this example, each unique gene is represented by at least two identical cDNAs on the array. The fold change value is calculated as described, then for each spot, a value above 5-fold change is accorded a code of 2, a fold-change value of less than 5 but greater than 2 is accorded a code of 1 and a fold-change value of less than 2 is accorded a code value of 0. A combined code is then derived by adding the code values for each identical cDNA on the array. The use of combined codes can greatly simplify the Cluster analysis and the subsequent visualisation (See Figure 14).

**10 Genes regulated 'early' in GM-CSF inhibition of neutrophil apoptosis are identified by Suppression Subtractive Hybridisation.**

This example describes the use of SSH, with modifications discussed, to identify and clone genes that are regulated 'early' in the process of GM-CSF inhibition of neutrophil apoptosis. Many of these DNA sequences are novel, i.e. they are not recognised by BLAST analysis to public gene sequence databases.

Following subtractions between 2 and 4 hour time points of GM-CSF treatment, with and without the presence of fungal metabolite Gliotoxin, a preliminary screening procedure is applied to arrays of clones generated from the subtracted libraries, (as described). Approximately 1000 clones are screened from each of the four subtracted libraries, yielding 700 differentially expressed clones.

The subtracted cDNA prepared by SSH may be used to probe the LifeGrid Arrays. Comparison of the data from this hybridization with that derived from hybridization with unsubtracted probe provides an indication of

1. the efficiency of the SSH subtraction
2. the gene representation within the subtracted cDNA
3. the overlap between SSH derived probes and probes prepared directly from cellular RNA

Using this approach we demonstrate differential expression of Superoxide Dismutase (Mn-SOD; as is previously identified using LifeGrid microarray filters), Hypoxia Inducible factor (HIF $\alpha$ ) and a number of other genes in both the SSH cDNA and the unsubtracted total RNA probes.

5

A sample of clones differentially expressed in these libraries are sequenced as described. A number of clones correspond to well-characterised genes, while many others are unique and do not have any identity ('Novel Clone) with any expressed sequence in the public databases (GenBank NR and dbEST). The sequence identities are presented in Table 3.

10

**EXAMPLE 5: GM-CSF inhibition of neutrophil apoptosis is associated with significant changes in global mRNA expression.**

15

This example describes microarray analysis of gene expression changes associated with GM-CSF inhibition of neutrophil apoptosis, using LifeGrid microarray filters. In addition, GM-CSF inhibition of neutrophil apoptosis is blocked by the fungal metabolite Gliotoxin.

20

In excess of 2,000 genes are differentially expressed. A significant number of genes are up-regulated following addition of GM-CSF (17 genes are increased 2-fold or more 2h following addition of GM-CSF, 76 are increased by 4h and 156 by 6h). These up-regulated genes may represent potential survival factor genes which block the apoptosis in neutrophils. Many of these genes that are up-regulated by GM-CSF are blocked by the fungal inhibitor gliotoxin. These genes are further implicated in the survival effects of GM-CSF and may be regulated via NF $\kappa$ B transcription factor or by signal transduction via NADPH oxidase. All 17 genes up-regulated by GM-CSF at 2h are blocked by Gliotoxin (see Table 4 A). Of 76 genes up-regulated by GM-CSF at 4h, 60 are blocked by Gliotoxin (see Table 4 B). Of 156 genes up-regulated by GM-CSF at 2h, 57 are blocked by Gliotoxin (see Table 4 C).

25  
30

A significant number of genes are down-regulated following addition of GM-CSF and these also represent candidate target genes (see Figure 15).

**Example 6: Cluster-analysis: Comparison of coordinate patterns of gene expression, by bioinformatic data analysis, using this model system, allows the identification of cell pathways and processes associated with apoptosis and survival.**

Primary human neutrophils are isolated and purified from peripheral blood of normal healthy individuals. Neutrophils are resuspended in serum containing culture medium together with GM-CSF (50U) at a concentration of  $2 \times 10^6$ /ml, and cultured for 0h (control), 2h, 4h and 6h at 37°C. Additionally the fungal metabolite Gliotoxin is included with the GM-CSF treatments to block the GM-CSF mediated inhibition of apoptosis. After these times, total cellular RNA is extracted and this used as the starting material for analysis of gene expression changes using the Incyte "LifeGrid" microarray. Following cluster analysis, a cluster of genes are identified that contained several genes involved in neutrophil survival and whose transcription is up - regulated upon GM-CSF treatment. This cluster, which included Bcl2-A1 (a 'known' antiapoptotic neutrophil BCL2 family member) is termed the 'survival' cluster (See Figure 15). Among these genes, one group that could be clearly identified are those genes whose protein products are involved in protecting the cell against an increased oxidative environment (Fig 16). Thus GM-CSF increased expression of mitochondrial superoxide dismutase (dismutates superoxide to hydrogen peroxide), catalase (converts  $H_2O_2$  to  $H_2O$  and  $O_2$ ) and ferritin (which sequesters iron thus limiting the Fenton reaction and preventing production of hyper-reactive hydroxyl radical) above control levels and this coincided with the ability of GM-CSF to prolong survival in neutrophils. Since all these gene products are attenuators of the oxidative cellular response it is indicative that GM-CSF protects the cell by reducing the harmful effects of reactive oxygen species.

30

On further analysis of this survival cluster, we identified increased transcription of the bifunctional enzyme phosphofructokinase-2 (PFK-2)/fructose 2,6 -bisphosphatase

(FBPase-2) which regulates the cellular concentration of fructose 2,6- bisphosphate and which in turn can regulate the function of phosphofructokinase-1 (See Figure 17). The ability of a cell to be able to reduce its oxidative environment is contingent on the cells ability to produce NADPH. This is particularly important in tissues that carry out the biosynthesis of fatty acids such as the liver and mammary tissue and other cells that are subjected to oxidative stress (Voehringer D.W., Hirschberg D.L., Xiao J., Lu Q., Roederer M., Lock, C.B. Herzenberg L.A., Steinman L., Herzenberg, L.A. (2000) PNAS, 97(6) pp2680-2685). Consequently, genes, which are represented on the array and whose products are known to be important in the pentose phosphate pathway are examined.

GM-CSF upregulates fructose 1,6 bisphosphatase (which functions to convert fructose 1,6 bisphosphate to fructose 6 phosphate which can then reversibly convert to Glucose-6-phosphate, the primary substrate for the pentose phosphate pathway. Simultaneously with up regulation of fructose 1,6 bisphosphatase, GM-CSF down regulated the transcription of phosphofructokinase 1 (whose role is to convert fructose 6 phosphate to fructose 1,6 bisphosphate), again ensuring that the pentose phosphate pathway is favoured above glycolysis. Therefore it is concluded that GM-CSF increases the formation of glucose 6 phosphate which can then be utilised in generation of reducing NADPH via the pentose phosphate pathway.

This survival cluster also contains a number of EST with as yet unknown function, these represent potentially novel target genes (see Figure 15).

**Example 7: In vitro cell based assays to confirm pro- and/or anti-apoptotic role for candidate genes identified by gene expression analysis.**

This example describes the establishment and use of a number of cell-based in vitro assays to measure pro- or anti-apoptotic activity by the ectopic overexpression of full-length cDNA coding sequence in a mammalian cell line; HeLa. For this example Mn superoxide dismutase is cloned and confirmed to have anti-apoptotic activity. Superoxide dismutase is identified by both microarray analysis and by SSH analysis of

gene expression, and is representative of genes found in survival cluster by this model system.

#### **Obtaining full-length cDNA sequence.**

5

In this example full length human SOD mRNA sequence is identified from the GenBank Acc# X07834. Full length cDNA coding sequence is amplified by RT-PCR from neutrophil RNA using standard techniques, and cloned into the expression vector pCR3.1-Uni (Invitrogen).

10

Alternatively, full-length clones are isolated from poly A<sup>+</sup> RNA using Smart<sup>TM</sup> RACE Technology (Clontech) according to the manufacturers protocol. Briefly, double stranded cDNA is generated using the Smart II oligonucleotide and the CDS Primer (both supplied in the kit). Specific PCR products are then generated using the PCR primer (supplied in the kit) and gene specific primers (sense and antisense primers generated from the DNA sequence to be extended). To increase specificity, nested sense and antisense primers are used in secondary PCR amplification. PCR products are then ligated into plasmids, such as the TA cloning system (Invitrogen), transformed into competent cells and expanded. Plasmids are purified using mini-prep isolation system (Qiagen) and plasmids are submitted to MWG for sequencing. Specific 5' and 3' sequences are identified using sense and antisense gene specific primers. Products are to be sequenced approximately 10 bases 5' of the initiation codon for 5' PCR products and 10 bases 3' of stop codons for 3' PCR products. Using sequence data, 5' and 3' primers are made and full-length cDNA is amplified.

25

#### **Transfection of HeLa cells to generate stable cell lines.**

HeLa cells are seeded ( $2 \times 10^6$ ) in a T75 tissue culture flask and incubated overnight at 37°C. 36µg plasmid DNA (containing candidate gene cDNA) are transfected overnight using Calfos (as described by manufacturer; Clontech). The Calfos reagent and DNA is removed, the cells washed with PBS and incubated in fresh medium overnight. The

30



following day G418 antibiotic (500µg/ml) is added and the cells incubated for one week, where after they are expanded for use in cell based validation assays.

**Determination of functional impact of ectopic expression of candidate gene on**  
5 **ciplatin-induced apoptosis.**

This example describes an assay to measure sensitivity or resistance to cisplatin-induced apoptosis in HeLa cells. Cell lines are generated by transfection, which stably express a candidate gene cDNA, in this example Mn superoxide dismutase. The cells  
10 are challenged with a cytotoxic agent, Cisplatin that induces apoptosis in HeLa in a dose dependent fashion.

Stable cell lines are seeded into wells of a 96-well microtitre plate at a concentration of 5000 cells per well. 4 wells of each cell line for each drug concentration are set up and  
15 plates incubated overnight. Cisplatin (0-200µg/ml) is added to each well and incubated for 24h, following which cell viability is measured by MTT.

Figure 18 shows that ectopic expression of superoxide dismutase in HeLa cells confers a dose dependent resistance to apoptosis induced by cisplatin, confirming that  
20 superoxide has anti-apoptotic function.

**Determination of functional impact of ectopic expression of candidate gene on**  
**p53-induced apoptosis.**

25 This example describes an assay to measure sensitivity or resistance to p53-induced apoptosis in HeLa cells. Cell lines are generated by transfection, which stably express a candidate gene cDNA, in this example Mn superoxide dismutase. EGFP-expressing cells are compared as a control. The cells are challenged by transfection and ectopic expression of a full-length cDNA for human p53 transcription factor that induces  
30 apoptosis in HeLa.

Stable cell lines are seeded into wells of a 24-well microtitre plate at a concentration of  $1.5 \times 10^6$  cells per well, and incubated overnight. A plasmid containing full length human p53 coding sequence (Acc# X54156) cloned in pCR3.1 expressing hygromycin antibiotic resistance is transfected into cells using Calfos reagents (according to  
5 manufacturers instructions, Invitrogen). Post-transfection cells are washed free of Calfos reagent and allowed to recover overnight and then selected for antibiotic resistance by the addition of 500 $\mu$ g/ml hygromycin. One week following antibiotic selection cells are washed twice to remove cell debris and stained with 1% crystal violet (in ethanol). Stain is released by extraction with 33% acetic acid and cell viability quantitated  
10 spectrophotometrically @570nm.

**Figure 19** shows that ectopic expression of superoxide dismutase in HeLa cells confers resistance to apoptosis induced by expression of the transcription factor p53.

15

All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and system of the present invention will be apparent to those skilled in the art without departing from the scope and spirit of the present invention. Although the present invention has  
20 been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in biochemistry, molecular biology and biotechnology or related fields are intended to be within the scope of the following  
25 claims.

49

Table 1

	2h	4h	6h	8h	20h	24h
Sample 1	6	11	9	44	91	83
Sample 2	7	15	12	48	-	93

5

Table 2.

Time	1 h	2h	3h	4h	5h	6h	7h
Sample 1	-	7.61	10.77	25.85	12.38	8.34	6.75
Sample 2	4.35	14.72	17.32	9.86	10.17	7.46	6.99

Table 3

Clone ID	Annotation
104 F8	Novel Clone
106 B7	NADH-ubiquinone oxoreductase chain 4
107 F5	NADH-ubiquinone oxoreductase chain 4
110 G6	Plasma glutamate carboxypeptidase (PGCP)
105 D12	Novel Clone
105 E11	Novel Clone
105 F2	Novel Clone
111 A7	Cytochrome B
111 F12	ATP Synthase A chain; F0 subunit 6
111 H12	Tomoregulin
113 A4	Hypoxia inducible factor 1 alpha
106 B4	Novel Clone
107 E3	Novel Clone
109 C2	Novel Clone
112 A4	IL8
112 B4	Novel Clone
113 F1	IL8
114 F7	IL8
105 D1	Novel Clone
105 F8	Novel Clone

Table 4

A.

UniGene Cluster	Annotation	T0h	Glio 2h	GM-CSF 2h
Hs.143212	cystatin F (leukocystatin)	0.59	0.64	1.63
Hs.78045	tissue factor pathway inhibitor 2	0.12	0.19	0.33
Hs.99932	diacylglycerol kinase, theta (110kD)	0.62	0.79	1.33
Hs.40968	heparan sulfate (glucosamine) 3-O-sulfotransferase 1	0.07	0.13	1
Hs.1499	heat shock transcription factor 1	0.1	0.09	0.4
Hs.111373	KIAA0423 protein	1.06	0.65	6
Hs.139120	ribonuclease P (30kD)	0.69	3.2	2.72
Hs.36977	hemoglobin, delta	0.21	0.29	0.55
Hs.5105	ESTs	0.22	0.16	0.45
Hs.153678	reproduction 8	2.12	0.99	4.41
Hs.920	modulator recognition factor I	0.75	0.57	2.19
Hs.13063	transcription factor CA150	0.15	0.18	0.36
Hs.813	ATPase, H <sup>+</sup> /K <sup>+</sup> exchanging, beta polypeptide	0.34	0.26	0.81
Hs.194724	pronapsin A	0.3	0.87	2.16
Hs.48295	RNA helicase family	0.57	0.05	2.91
Hs.129078	ESTs	0.24	0.41	0.64

B.

UniGene Cluster	Annotation	T0h	Glio 4h	GM-CSF 4h
Hs.227817	BCL2-related protein A1	0.1	0.18	0.72
Hs.12107	putative breast adenocarcinoma marker (32kD)	1.11	1.39	2.49
Hs.101414	KIAA0557 protein	1.61	2.66	5.1
Hs.108014	tubulin, beta, 5	0.06	0.06	0.31
Hs.109281	Nef-associated factor 1	1.18	0.98	3.03
Hs.76307	neuroblastoma candidate region, suppression of tumorigenicity 1	2.96	1.66	9.01
Hs.123233	ESTs	0.54	0.25	1.5
Hs.248038	major histocompatibility complex, class I, C	5.11	5.6	17.16
Hs.44512	ESTs	7.52	15.44	17.07
Hs.159494	Bruton agammaglobulinemia tyrosine kinase	0.17	0.13	0.4
Hs.104624	aquaporin 9	0.29	0.17	0.67
Hs.44197	Homo sapiens mRNA; cDNA DKFZp564D0462 (from clone DKFZp564D0462)	2	3.48	4.15
Hs.80706	diaphorase (NADH/NADPH) (cytochrome b-5 reductase)	4.99	6.34	13.79
Hs.167740	butyrophilin, subfamily 3, member A1	1.25	2.09	2.69
Hs.77897	pre-mRNA splicing factor SF3a (60kD), similar to S. cerevisiae PRP9 (spliceosome-associated protein 61)	3.61	4.78	7.85

UniGene Cluster	Annotation	T0h	Glio 4h	GM-CSF 4h
Hs.198278	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4	0.12	0.25	1.13
Hs.75607	myristoylated alanine-rich protein kinase C substrate (MARCKS, 80K-L)	3.86	2.93	8.8
Hs.169610	CD44 antigen (homing function and Indian blood group system)	0.8	0.89	1.64
Hs.44070	ESTs	12.57	14.71	43.43
Hs.56562	ESTs	10.45	18.98	28.91
Hs.239138	pre-B-cell colony-enhancing factor	1.02	0.87	2.41
Hs.177781	superoxide dismutase 2, mitochondrial	0.61	1.02	5.28
Hs.111373	KIAA0423 protein	1.33	0.9	5.03
Hs.104481	Homo sapiens mRNA for Nck, Ash and phospholipase C gamma-binding protein NAP4, partial cds	6.42	13.83	14.54
Hs.242908	lecithin-cholesterol acyltransferase	3.69	5.74	11.01
Hs.153	ribosomal protein L7	0.03	0.28	0.71
Hs.179600	TAP binding protein (tapasin)	1.82	1.38	5.64
Hs.2561	nerve growth factor, beta polypeptide	0.41	0.35	2.36
Hs.82212	CD53 antigen	2.38	3.13	5.29
Hs.150580	putative translation initiation factor	1.76	1.73	5.1
Hs.2064	vimentin	0.66	0.5	2.11
Hs.81361	heterogeneous nuclear ribonucleoprotein A/B	14.18	4.04	35.31
Hs.119252	tumor protein, translationally-controlled 1	1.95	1.5	9.33
Hs.75372	N-acetylgalactosaminidase, alpha-	1.08	1.79	2.49
Hs.68061	ESTs, Weakly similar to sphingosine kinase [M.musculus]	0.4	0.43	1.08
Hs.74122	caspase 4, apoptosis-related cysteine protease	0.73	0.67	1.47
Hs.18350	chromosome 21 open reading frame 1	2.81	3.94	7.37
Hs.2488	lymphocyte cytosolic protein 2 (SH2 domain-containing leukocyte protein of 76kD)	1.06	0.78	2.3
Hs.60177	KIAA0996 protein	3.8	7.44	7.75
Hs.184592	KIAA0344 gene product	2.23	4.17	10.16
Hs.76452	C-reactive protein, pentraxin-related	0.36	0.15	0.78
Hs.753	formyl peptide receptor 1	1.03	1.01	2.44
Hs.33540	ESTs, Weakly similar to KIAA0765 protein [H.sapiens]	5.26	3.08	11.53
Hs.79018	chromatin assembly factor 1, subunit A (p150)	34.29	65.93	81.83
Hs.22176	ESTs	118.49	78.07	260.42
Hs.51077	integrin, alpha X (antigen CD11C (p150), alpha polypeptide)	2.62	4.09	6.92
Hs.5392	Homo sapiens clone 25030 mRNA sequence	0.7	0.72	4
Hs.813	ATPase, H+/K+ exchanging, beta polypeptide	0.34	0.3	3.15
Hs.62954	ferritin, heavy polypeptide 1	0.24	0.25	2.1

UniGene Cluster	Annotation	T0h	Glio 4h	GM-CSF 4h
Hs.239124	ESTs	1.88	4.61	3.96
Hs.194724	pronapsin A	0.3	0.44	5.97
Hs.21812	ESTs	3.4	6.45	6.87
Hs.129078	ESTs	0.24	0.38	1.12

C.

UniGene Cluster	Annotation	T0h	Glio T6h	GM-CSF T6h
Hs.143212	cystatin F (leukocystatin)	0.59	0.17	1.93
Hs.90370	actin related protein 2/3 complex, subunit 1A (41 kD)	0.08	0.13	0.34
Hs.10762	ESTs	0.23	0.23	0.49
Hs.78045	tissue factor pathway inhibitor 2	0.12	0.21	0.34
Hs.24930	tubulin-specific chaperone a	0.09	0.09	0.47
Hs.227817	BCL2-related protein A1	0.08	0.15	0.33
Hs.17518	Homo sapiens cig5 mRNA, partial sequence	0.58	0.5	1.23
Hs.109281	Nef-associated factor 1	1.18	0.97	3.01
Hs.76807	Human HLA-DR alpha-chain mRNA	0.24	0.29	0.53
Hs.44287	ESTs	0.26	0.28	0.9
Hs.104624	aquaporin 9	0.24	0.26	0.55
Hs.31314	retinoblastoma-binding protein 7	0.13	0.17	0.35
Hs.114138	ESTs	2.72	7.41	9.22
Hs.194562	telomeric repeat binding factor (NIMA-interacting) 1	0.26	0.28	0.67
Hs.208819	ESTs	0.06	0.04	0.64
Hs.82128	5T4 oncofetal trophoblast glycoprotein	0.85	0.57	1.81
Hs.19126	src kinase-associated phosphoprotein of 55 kDa	0.13	0.33	0.3
Hs.198278	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4	0.12	0.11	0.48
Hs.154583	RNA-binding protein S1-1, human homolog of	0.17	0.21	0.44
Hs.72964	makorin, ring finger protein, 3	0.15	0.2	0.44
Hs.133554	ESTs	0.37	0.5	0.76
Hs.159509	alpha-2-plasmin inhibitor	0.04	0.04	0.3
Hs.98183	ESTs	0.15	0.22	0.4
Hs.41683	cartilage paired-class homeoprotein 1	0.15	0.25	0.4
Hs.155995	KIAA0643 protein	0.17	0.3	0.4
Hs.2062	vitamin D (1,25- dihydroxyvitamin D3) receptor	0.61	0.87	1.39
Hs.51299	NADH dehydrogenase (ubiquinone) flavoprotein 2 (24kD)	0.36	0.3	0.97
Hs.170180	sialyltransferase 8 (alpha-2, 8-	0.16	0.14	0.84



UniGene Cluster	Annotation	T0h	Glio T6h	GM-CSF T6h
	polysialyltransferase) D			
Hs.177781	superoxide dismutase 2, mitochondrial	0.61	0.67	3.19
Hs.75789	N-myc downstream regulated	0.18	0.19	0.36
Hs.179600	TAP binding protein (tapasin)	1.82	1.48	3.69
Hs.100407	programmed cell death 4	0.39	0.56	0.81
Hs.103755	receptor-interacting serine-threonine kinase 2	0.19	0.08	0.4
Hs.123141	Wiskott-Aldrich syndrome-like	0.11	0.12	0.51
Hs.2064	vimentin	0.66	0.66	1.48
Hs.119252	tumor protein, translationally-controlled 1	1.77	2.22	4.35
Hs.13957	ESTs	0.24	0.26	0.51
Hs.227133	KIAA0670 protein	0.13	0.08	0.3
Hs.28169	KIAA0459 protein	0.77	0.68	2.55
Hs.108947	KIAA0050 gene product	0.31	0.08	1.47
Hs.68061	ESTs, Weakly similar to sphingosine kinase [M.musculus]	0.4	0.4	1.01
Hs.44426	ESTs, Weakly similar to PHOSPHOLIPID HYDROPEROXIDE GLUTATHIONE PEROXIDASE [H.sapiens]	0.59	0.67	1.39
Hs.251972	complement component 3	0.21	0.22	0.46
Hs.927	myosin-binding protein H	0.17	0.31	0.34
Hs.13063	transcription factor CA150	0.1	0.26	0.3
Hs.113216	oxytocin, prepro- (neurophysin I)	0.13	0.04	0.31
Hs.62954	ferritin, heavy polypeptide 1	0.22	0.22	1.24
Hs.194724	pronapsin A	0.3	0.16	0.8
Hs.235069	RecQ protein-like (DNA helicase Q1-like)	1.16	2.23	2.71
Hs.124380	ESTs	0.52	0.57	1.8
Hs.241510	interferon-induced protein 41, 30kD	0.35	0.34	0.72
Hs.129078	ESTs	0.24	0.14	1.16

CLAIMS

1. A method for identifying a gene product which modulates the transition of a  
5 cell between a non-apoptotic state and an apoptotic state, comprising the steps  
of:  
(a) exposing the cell to an inhibitor of GM-CSF mediated inhibition of  
apoptosis; and  
(b) exposing the cell to one or more agents which increase tyrosine  
10 phosphorylation; and  
(c) placing the cell in conditions which permit it to undergo spontaneous  
apoptosis; and  
(d) monitoring the level(s) of expression of the one or more gene products in  
the cell; and  
15 (e) identifying gene product(s) whose expression has been increased, decreased  
or modified as a result of performing steps (a) to (d).
2. A method according to claim 1, further comprising the step of determining the  
20 level(s) of expression of one or more gene product(s) in a cell to establish a  
reference expression level;
3. A method according to claim 1 or claim 2 wherein one or more agent/s which  
increases tyrosine phosphorylation is selected from the group consisting of:  
phenylarsine oxide (PAO), Genestein.  
25
4. A method according to any preceding claim wherein step (b); that is, exposing  
the cell to one or more agent/s which increases tyrosine phosphorylation is  
substituted by the step of; exposing the cell to GM-CSF.

5. A method according to any preceding claim wherein one or more inhibitor in  
step (a) is Gliotoxin.
6. A method according to any one of claims 1 to 4 wherein one or more inhibitors  
5 in step (a) is an agent which increases the level of NFkB within a cell.
7. A method according to claim 6 wherein one or more agents is any one of: LPS,  
TNF $\alpha$ , p60 component/s of NFkB, p50 component/s of NFkB.
- 10 8. A method according to any one to claims 1 to 4 wherein the inhibitor in step (a)  
is one or more agent/s which inhibits the functions of NADPH oxidase.
9. A method according to claim 8 wherein one or more agents is any one of:  
phenylarsine oxide, diphenylene iodonium.
- 15 10. A method according to any preceding claim wherein the expression levels are  
determined by assessing polypeptide production.
11. A method according to any one of claims 1 to 9, wherein expression levels are  
20 determined by assessing polypeptide post-translational modification.
12. A method according to any one of claims 1 to 9, wherein expression levels are  
determined by assaying gene-transcription.
- 25 13. A method according to any preceding claim, wherein the cell is selected from  
the group consisting of neutrophils, cells with neutrophil characteristics such as  
HL60 cells, and HeLA cells.
14. A method according to claim 13 wherein the cell is cultured in the presence of  
30 an inhibitor of GM-CSF mediated inhibition of apoptosis.

15. A method according to any preceding claim wherein the onset of apoptosis is monitored by morphological analysis, externalisation of membrane phospholipid phosphatidyl serine or caspase activation analysis.
- 5 16. A method according to claim 12, wherein the expression levels of a plurality of gene product/s are determined by hybridisation of one or more mRNA populations to a set of polynucleotides arrayed on to a substrate.
- 10 17. A method of according to claim 10 or claim 11 wherein the expression levels of a plurality of gene product/s are determined by 2D-polyacrylamide gel electrophoresis of one or more polypeptide populations.
- 15 18. A method according to any preceding claim wherein the expression levels of the gene product/s are determined by analysis of global gene expression patterns.
19. A method according to claim 18 wherein global gene expression is analysed using microarray or SSH.
- 20 20. A method according to any preceding claim which is configured to identify gene products which inhibit the induction of apoptosis.
21. The use of gliotoxin to inhibit the GM-CSF mediated inhibition of apoptosis.
- 25 22. A system for modelling GM-CSF induced apoptosis in a cell comprising the steps of:
- (a) the provision of a population of cells;
  - (b) exposing the cell to an inhibitor of CM-CSF mediated inhibition of apoptosis; and
  - 30 (c) exposing the cell to an agent which increases tyrosine phosphorylation and/or; exposing the cell to CM-CSF; and

(d) placing the cell in conditions which allow it to undergo spontaneous apoptosis; and

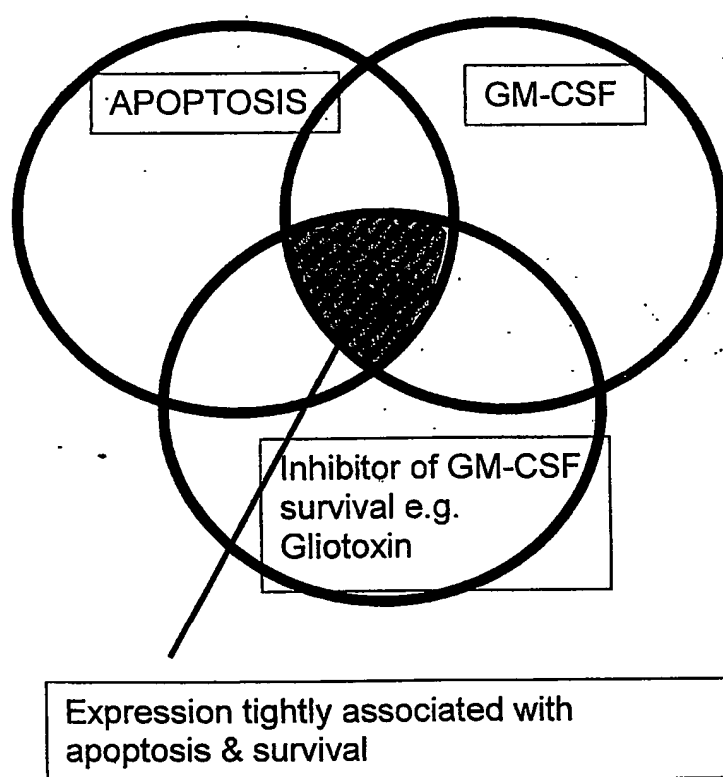
(e) analysing the gene expression of the cell population; and

(f) assessing the onset of apoptosis in said cell population.

5

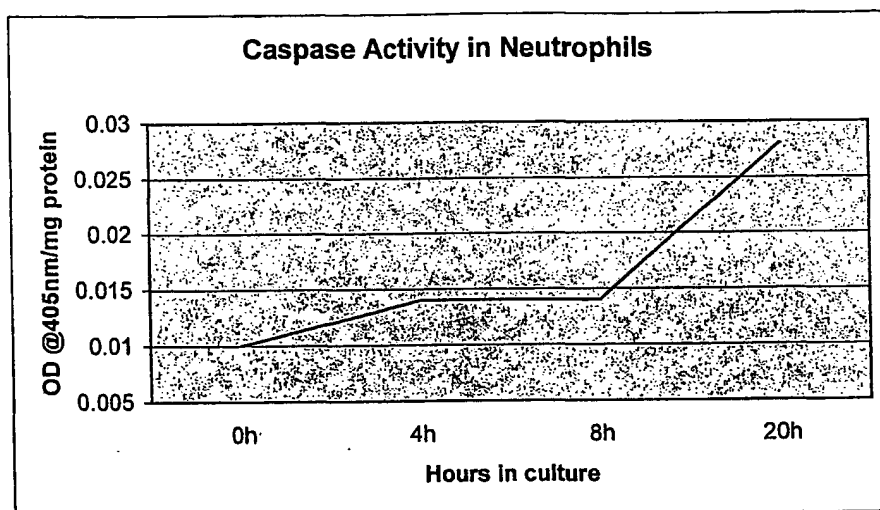
23. A system according to claim 22 wherein the cells are selected from the group consisting of neutrophils, HL60 cells and HeLa cells.
24. A system according to claim 22 having any one or more of the features of claims 1 to 21.
- 10

FIGURE 1



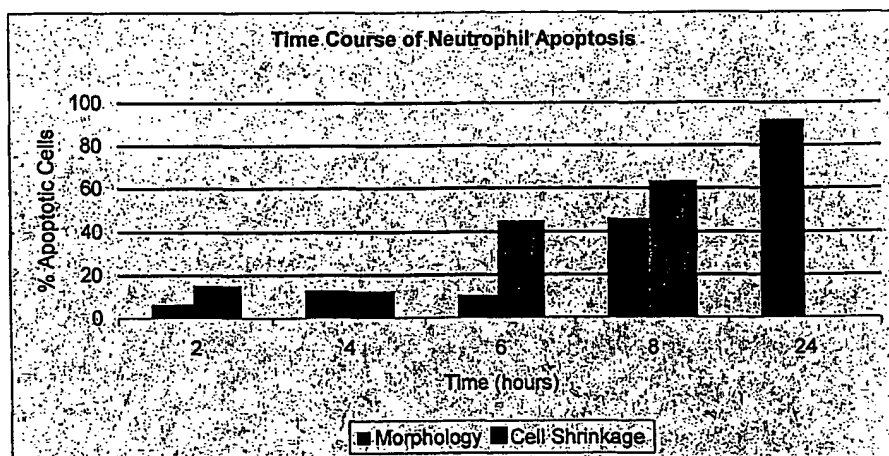
2/19

FIGURE 2



3/19

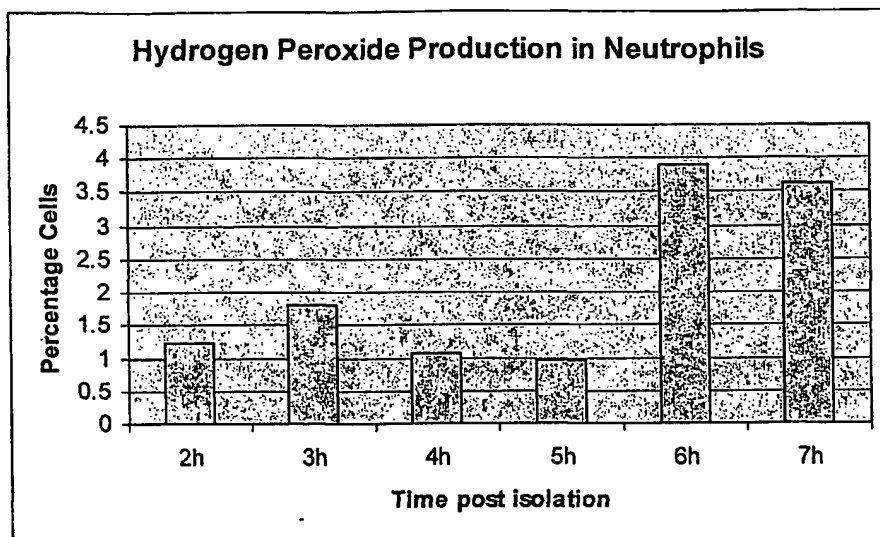
Figure 3





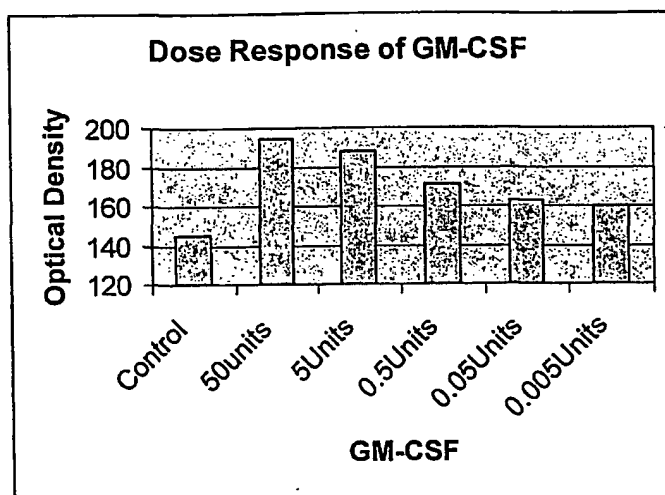
4/19

Figure 4



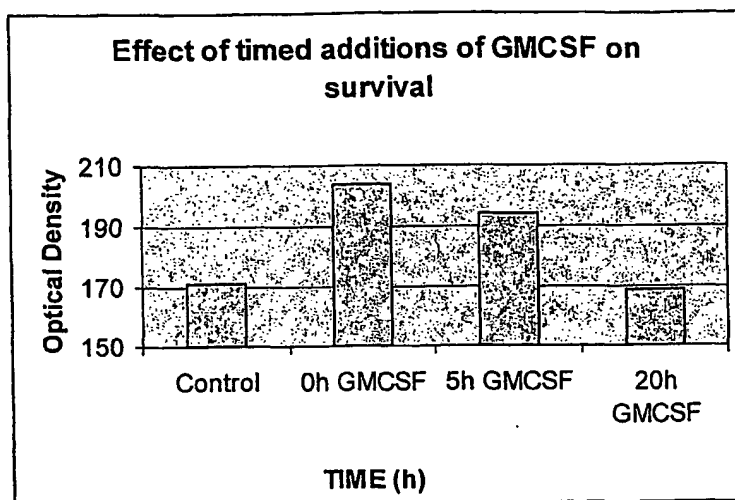
5/19

Figure 5



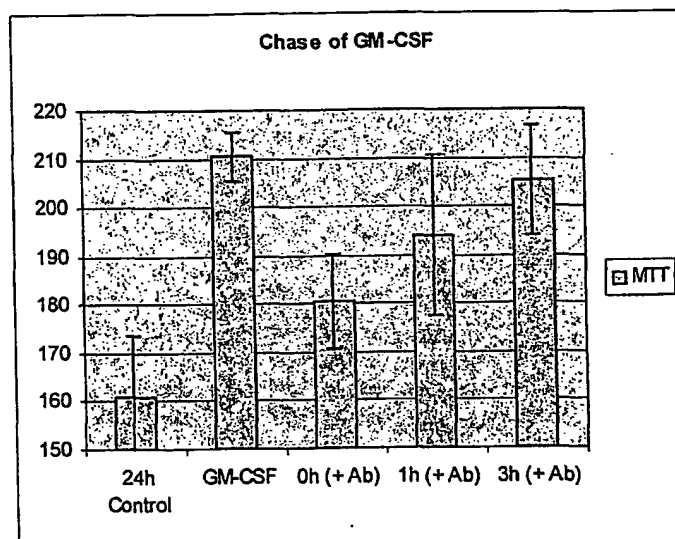
6/19

Figure 6



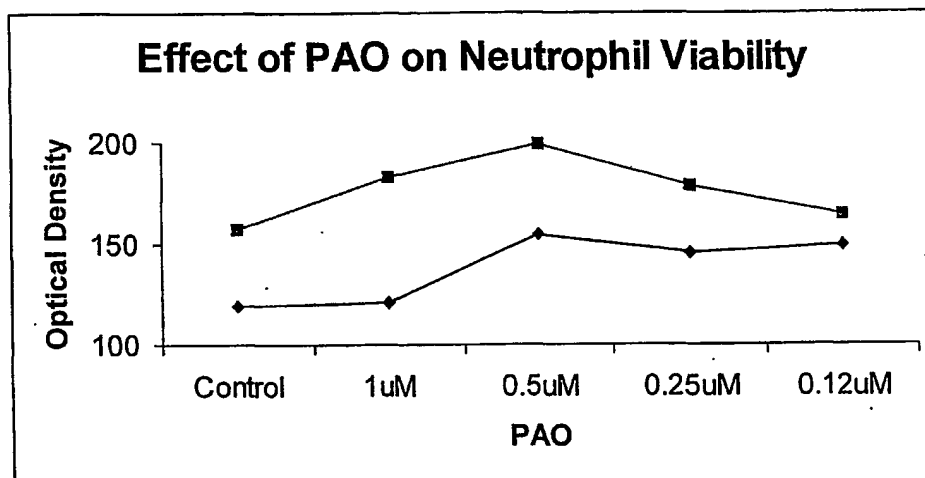
7/19

Figure 7



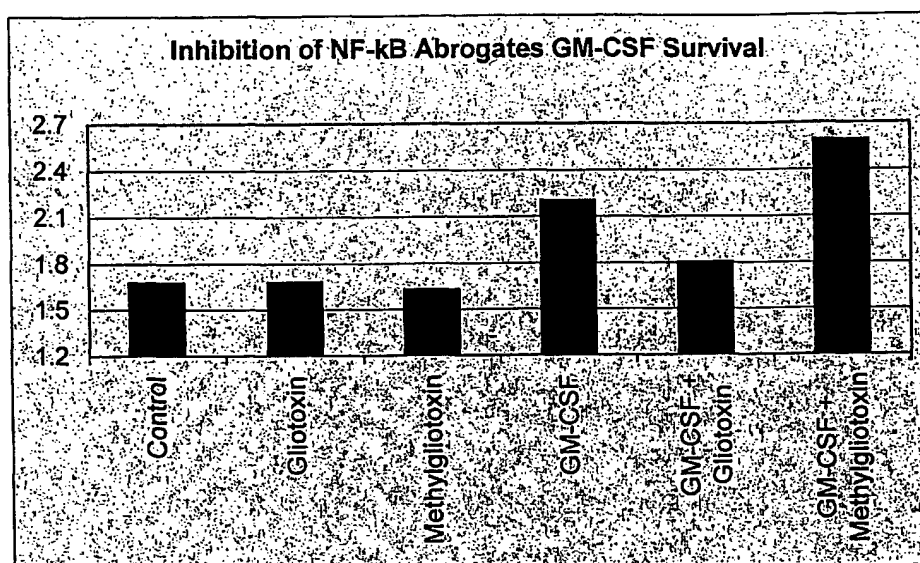
8/19

Figure 8



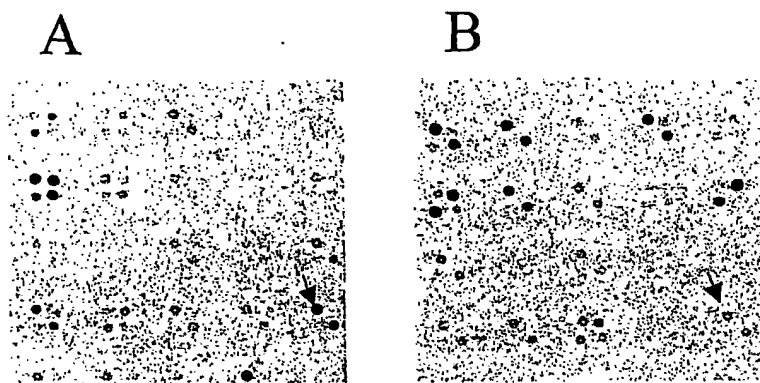
9/19

FIGURE 9



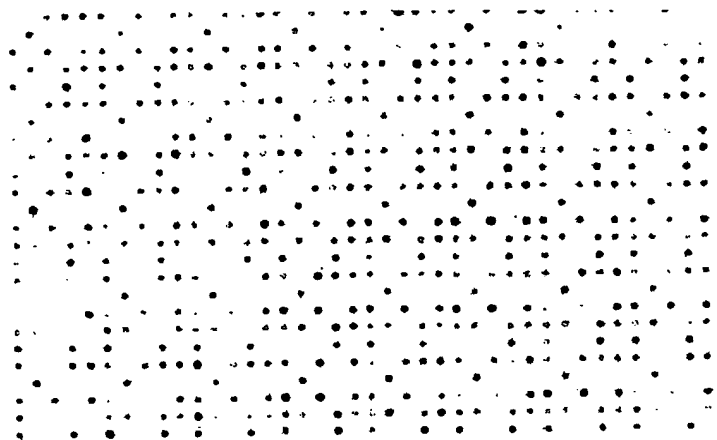
10/19

Figure 10



11/19

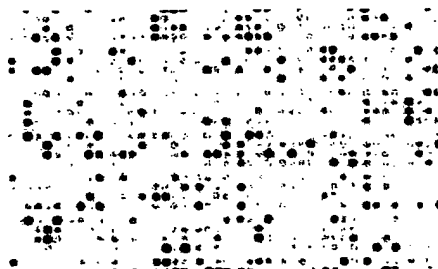
Figure 11





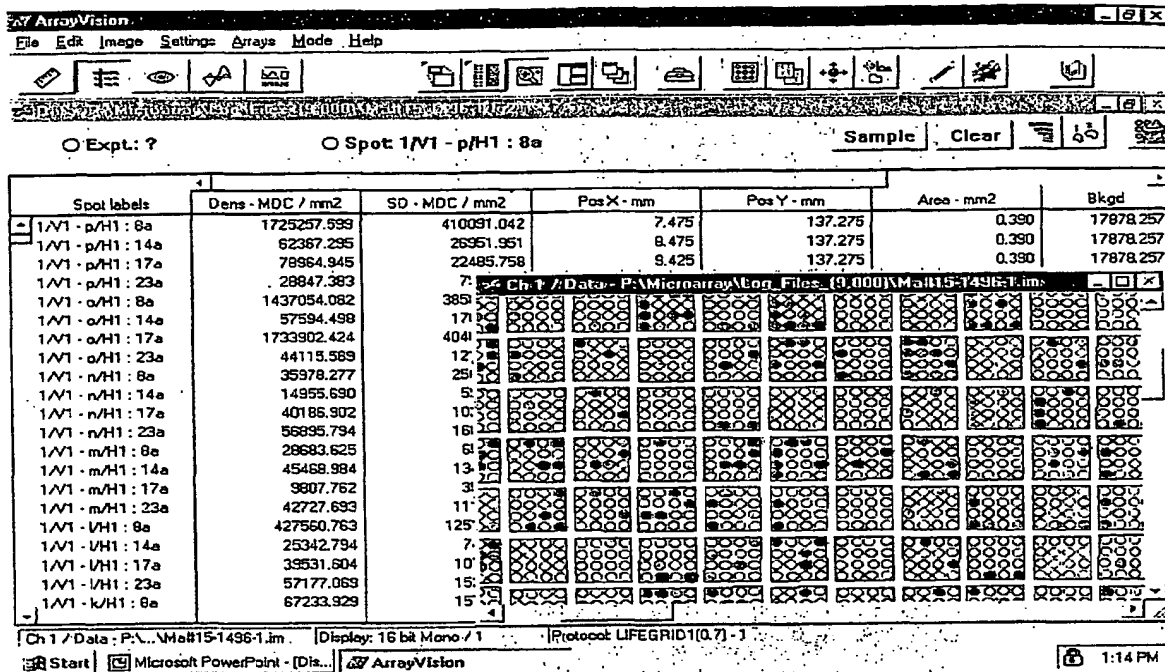
12/19

Figure 12



13/19

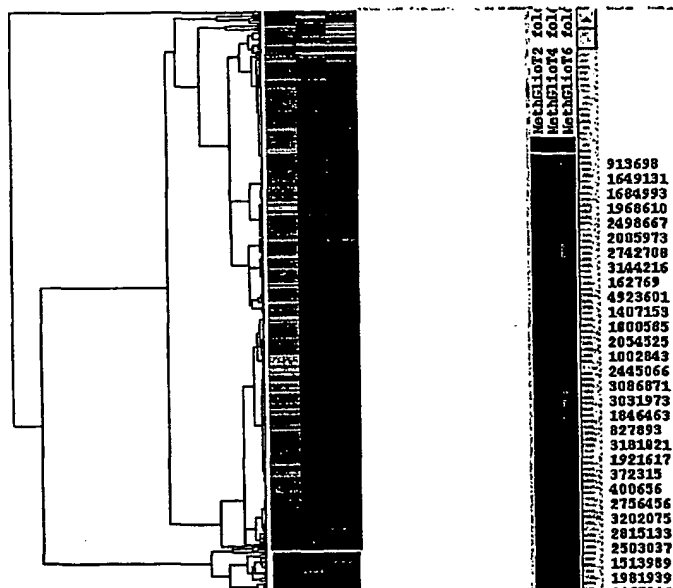
Figure 13



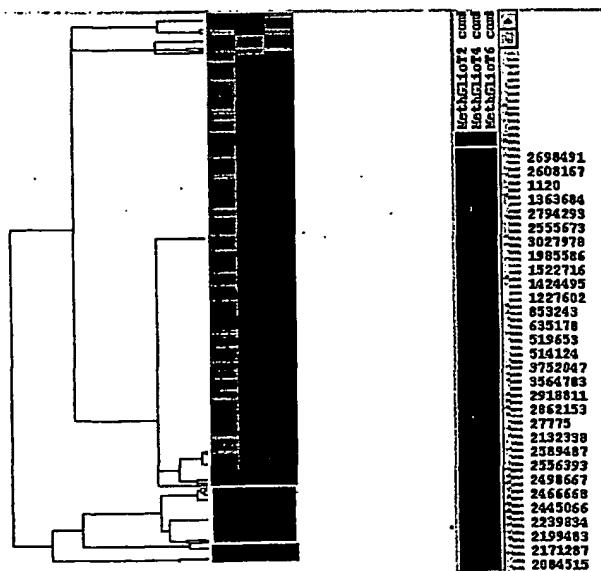
14/19

Figure 14

A

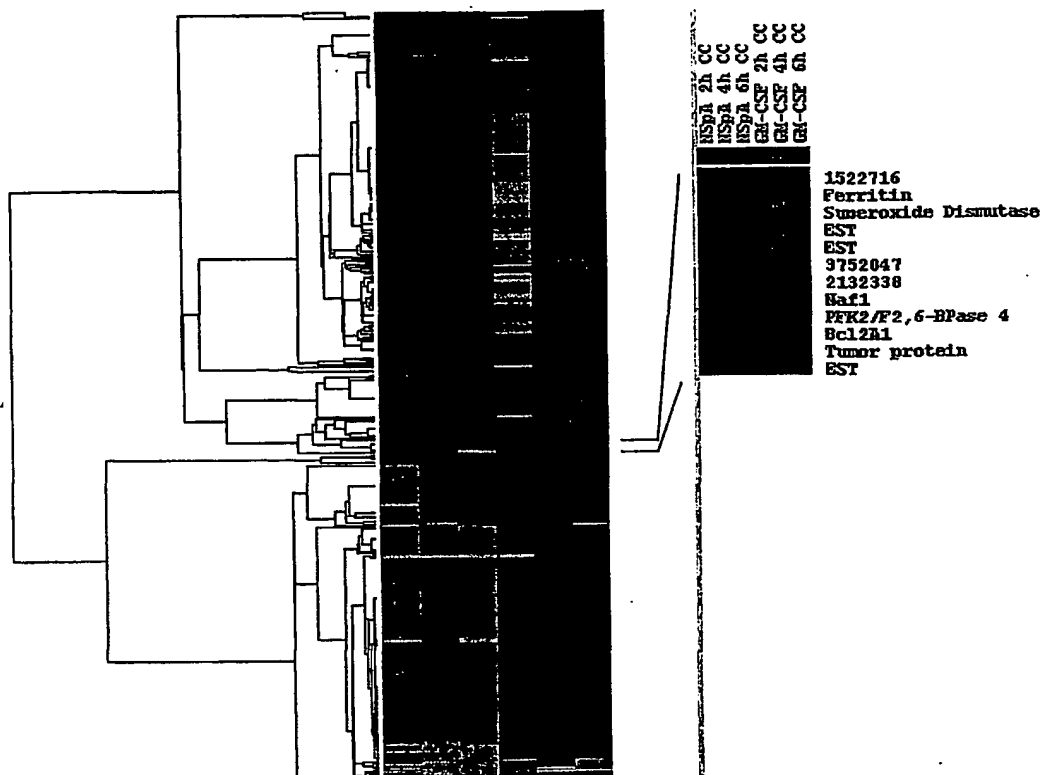


B



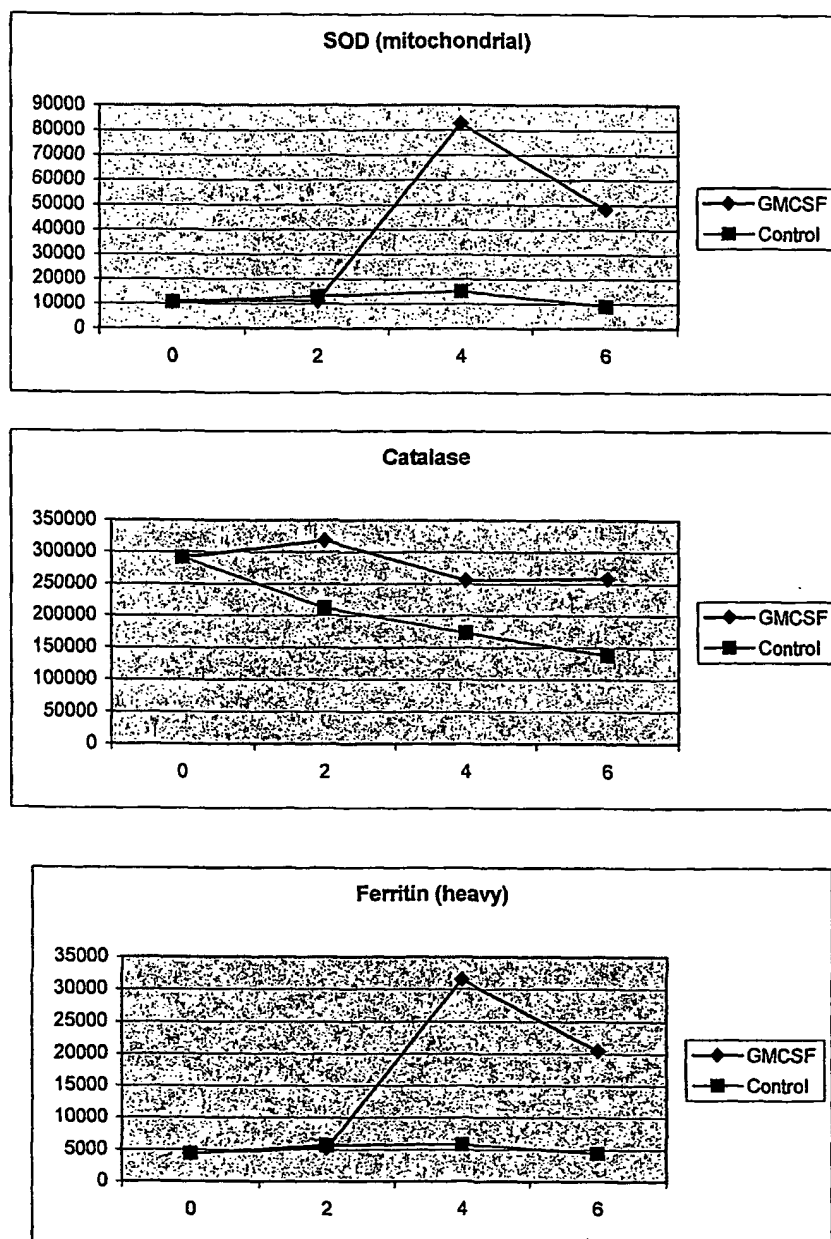
15/19

Figure 15



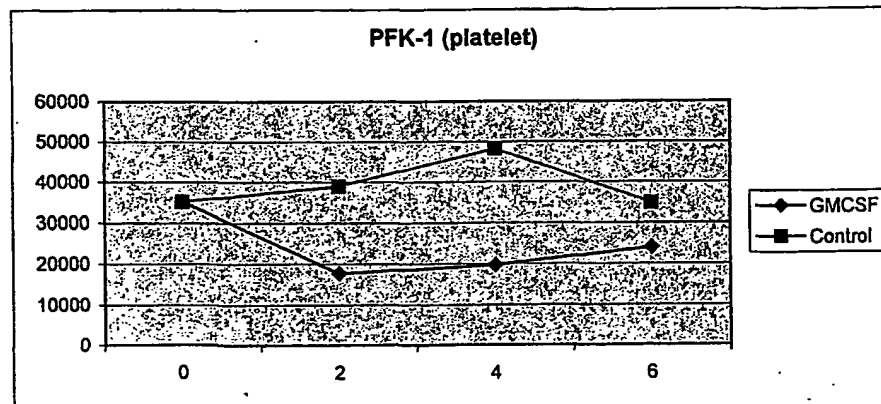
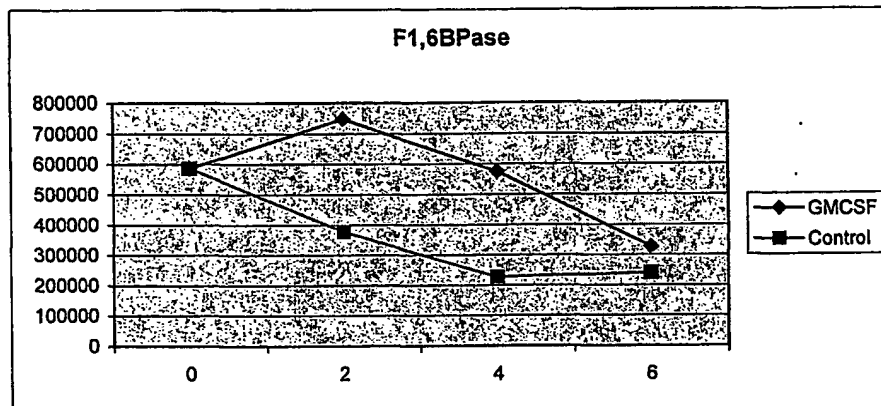
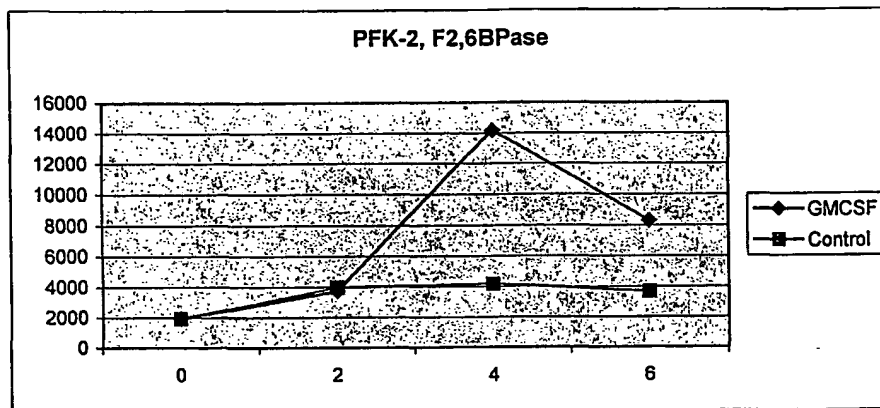
16/19

Figure 16



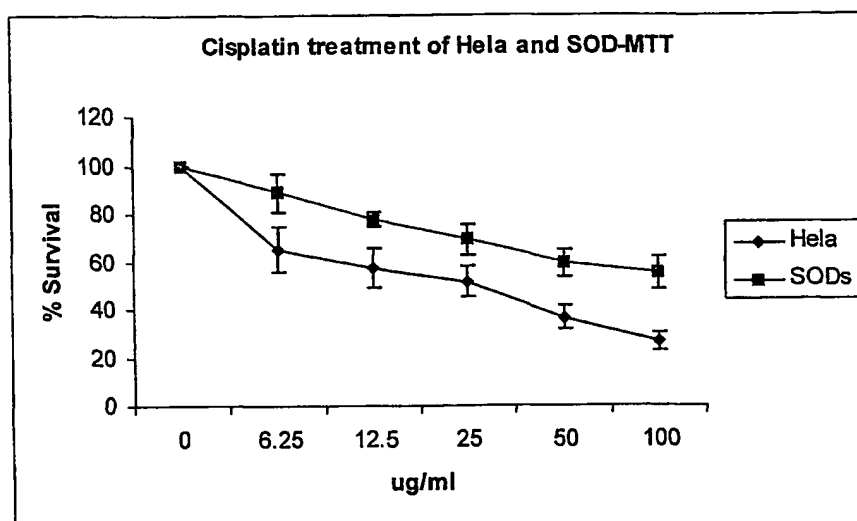
17/19

Figure 17



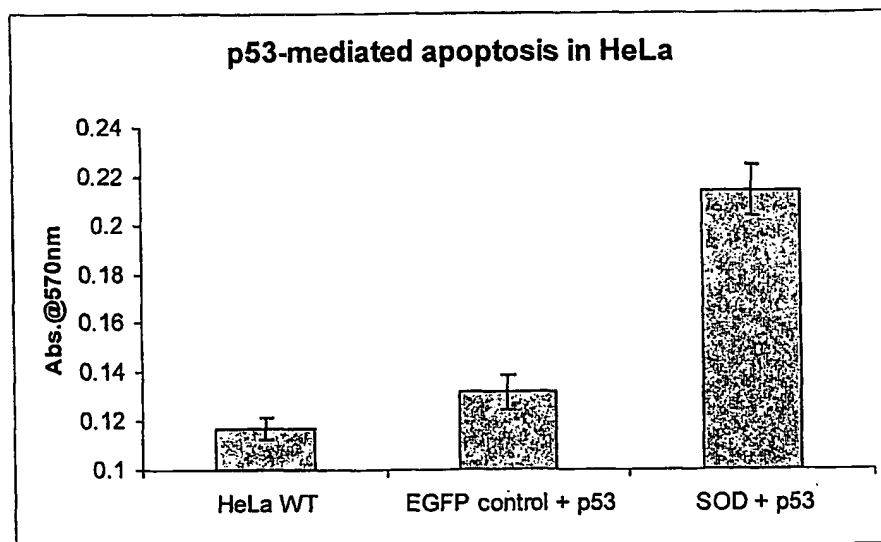
18/19

FIGURE 18



19/19

Figure 19





(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
17 January 2002 (17.01.2002)

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(25) Filing Language: **English**

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(71) Applicant (for all designated States except US): **EIRX THERAPEUTICS LIMITED [IE/IE]**; Cork Airport Business Park, Kinsale, Cork (IE).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **COTTER, Tom [IE/IE]**; EIRx Therapeutics Limited, Cork Airport Business Park, Kinsale Road, Cork (IE). **HAYES, Ian [GB/GB]**; EIRx Therapeutics Ltd, Cork Airport Business Park, Kinsale Road, Cork (IE). **MURPHY, Finbarr [IE/IE]**;

EIRx Therapeutics Ltd, Cork Airport Business Park, Kinsale Road, Cork (IE). **SEERY, Liam [IE/IE]**; EIRx Therapeutics Ltd, Cork Airport Business Park, Kinsale Road, Cork (IE).

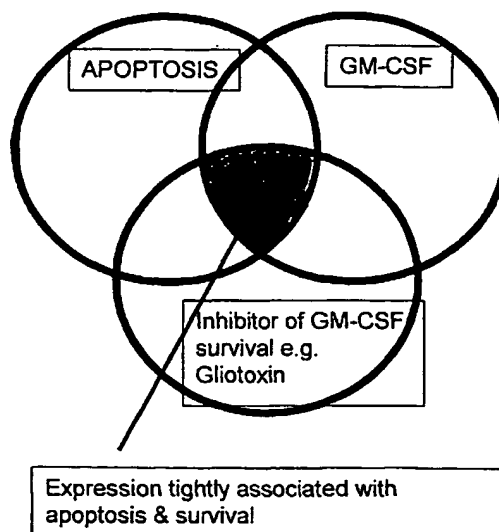
(74) Agents: **MASCHIO, Antonio et al.**; D Young & Co., 21 New Fetter Lane, London EC4A 1DA (GB).

(81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

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[Continued on next page]

(54) Title: **SCREENING METHOD**



(57) Abstract: The present invention relates to a method for identifying a gene product which modulates the transition of a cell between a non-apoptotic state and an apoptotic state, comprising the steps of: (a) exposing the cell to an inhibitor of GM-CSF mediated inhibition of apoptosis; and (b) exposing the cell to one or more agents which increase tyrosine phosphorylation; and (c) placing the cell in conditions which permit it to undergo spontaneous apoptosis; and (d) monitoring the level(s) of expression of the one or more gene products in the cell; and (e) identifying gene product(s) whose expression has been increased, decreased or modified as a result of performing steps (a) to (d).



**Declaration under Rule 4.17:**

— *of inventorship (Rule 4.17(iv)) for US only*

**Published:**

— *with international search report*

**(88) Date of publication of the international search report:**

16 January 2003

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

tional Application No  
PCT/GB 01/03101

According to International Patent Classification (IPC) or to both national classification and IPC

Minimum documentation searched (classification system followed by classification symbols)  
IPC 7 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal. BIOSIS

### C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Y. WANG, T. REA, J. BIAN, S. GRAY, Y. SUN: "Identification of genes responsive to etoposide-induced apoptosis: application of DNA chip technology" FEBS, vol. 445, 1999, pages 269-273, XP002209541 the whole document	1-19, 21-24
A	W. YUGANG, L. HONTAO, Z. YINGMEI, M. DALONG: "cDNA cloning and expression of an apoptosis-related gene, human TFAR15 gene" SCIENCE IN CHINA SERIES C LIFE SCIENCES, vol. 42, no. 3, 1999, pages 323-329, XP002209542 the whole document	1-19, 21-24

☒ Further documents are listed in the continuation of box C.

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Date of the actual completion of the international search

19 August 2002

Date of mailing of the international search report

18/09/2002

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Jacques, P

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International Application No  
PCT/GB 01/03101

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	COXON ANGELA ET AL: "Cytokine-activated endothelial cells delay neutrophil apoptosis in vitro and in vivo: A role for granulocyte/macrophage colony-stimulating factor" JOURNAL OF EXPERIMENTAL MEDICINE, TOKYO, JP, vol. 190, no. 7, 4 October 1999 (1999-10-04), pages 923-933, XP002194526 ISSN: 0022-1007 the whole document	1-19, 21-24
A	S.J. WILSON, B.A. LEONE, D. ANDERSON, A. MANNING, S.T. HOLGATE: "Immunohistochemical analysis of the activation of NF-kappaB and expression of associated cytokines and adhesion molecules in human models of allergic inflammation" JOURNAL OF PATHOLOGY, vol. 189, 1999, pages 265-272, XP002209544 the whole document	5-7, 21
A	P. WEINMANN, P. GAEHTGENS, B. WALZOG: "Bcl-X1 and Bax-alpha mediated regulation of Apoptosis of human neutrophils via caspase-3" BLOOD, vol. 93, no. 9, 1999, pages 3106-3115, XP002209559 the whole document	1-19, 21-24
A	MCCUBREY J.A.: MAY W. STRATFORD, DURINO V. MUFSON A.: "serine/threonine phosphorylation in cytokine signal transduction" LEUKEMIA, vol. 14, no. 1, January 2000 (2000-01), pages 9-21, XP002209543 the whole document	1-19, 21-24
A	WO 99 32514 A (WARNER LAMBERT CO ;SUN YI (US)) 1 July 1999 (1999-07-01) page 13, line 25 -page 14, line 24	1-19, 21-24
A	EP 0 926 242 A (AJINOMOTO KK) 30 June 1999 (1999-06-30) the whole document	5-7, 21

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/GB 01/03101

## Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

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because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos.: 20  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:  
see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this International application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

## FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 20

Present claim 20 relates to an extremely large number of possible methods. In fact, the claim contain so many options that a lack of clarity within the meaning of Article 6 PCT arises to such an extent as to render a meaningful search of the claims impossible. Consequently, the search has been carried out for those parts of the application which do appear to be clear, namely claims 1-19 and 21-24. it would appear that the method of claim 20 is not characterised by any technical feature/procedural step which makes it difficult if not impossible to determine the matter for which protection is sought.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

## INTERNATIONAL SEARCH REPORT

Information on patent family members

national Application No

PCT/GB 01/03101

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